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Inhibition of angiogenesis has been shown to be an effective strategy in cancer therapy in mice. We constructed a recombinant adenovirus that expresses endostatin, which resulted in a significant delay of tumor progression of JC breast and Lewis lung carcinoma, and more importantly, in complete prevention of lung metastases formation in Lewis lung carcinoma. The inability to control pre-established tumors may be due to insufficient circulating endostatin levels or to inadequate local endostatin concentrations, both of which have been shown to be important for the anti-tumor effect of endostatin. Thus, we constructed a recombinant adenovirus expressing a murine Ig-endostatin fusion protein resulting in significantly higher circulating endostatin levels with improved anti-tumor activity. Furthermore, a tumor-targeted version of endostatin was made using homing peptides to activated endothelial cells (**RGD, NGR**) to increase directly endostatin concentrations in the tumor. Finally, conditionally replicating adenovirus (CRAD) targeted to the activated endothelium was very efficacious in selectively destroying 3-D capillary networks. In conclusion, the present study clearly demonstrates the potential of vector-mediated antiangiogenic gene therapy in cancer. Changes in vector design resulting in higher transgene expression levels, tumor-targeted delivery of endostatin, or endothelial selective replication of adenovirus may prove to be an effective anti-cancer therapy.

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Introduction

In recent years it has become clear that angiogenesis plays a pivotal role in tumor progression and metastases formation [1]. The target of antiangiogenic cancer treatment is the genetically normal endothelial cell. Therefore, the development of resistance to angiostatic therapy is very unlikely and has not been reported so far [2]. If a cancer exceeds the size of approximately 1-2 mm^3, recruitment of new blood vessels is needed (angiogenesis) to prevent tumor cell apoptosis. Therefore, continuous overexpression of antiangiogenic factors by gene therapy, for instance, should counteract the tumor-induced angiogenesis. We thus developped an antiangiogenic gene transfer approach to treat metastatic breast cancer using the potent angiostatic molecule endostatin. As it is known that local concentrations of angiogenesis inhibitors play a critical role in their anti-tumor efficacy we tested several different approaches to increase both serum levels of endostatin as well as direct delivery of endostatin to the activated tumor endothelium using tumor homing peptides or conditionally replicating adenovirus (CRAD).
Synergistic anti-tumor effects are expected when an anti-angiogenic approach to tumor treatment is used in conjunction with immunomodulatory therapy, such as IL-12.

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Body

The gutless adenovirus system was being developed in close collaboration with the consultant on this grant: Dr. Nuria Morral. The appropriate E1 minus vector with flanking loxP sites was constructed as well as a 293 cell line stably expressing Cre recombinase. After several rounds of amplification of gutless vectors, however, the helper contamination is still above (>1%) the acceptable target (< 0.1%) secondary to insufficient Cre recombinase expression of the packaging cell line. Attempts are being made to isolate a clone with higher Cre recombinase expression.

Meanwhile to prove the efficacy and also to test for toxicity of antiangiogenic gene therapy for breast cancer, we decided to construct an E1 minus recombinant adenovirus expressing endostatin under the control of the human EF-1α promoter. This construct was successfully used for *in vitro* and *in vivo* experiments in tumor bearing mice. These results were reported in detail in a publication [3]; enclosed in Appendix. The data clearly show the efficacy of endostatin gene therapy for cancer. A significant delay in tumor growth and total prevention of metastases formation was observed after systemic delivery of adenovirus expressing murine endostatin. Tumor regression, however, could not be documented. The results of our study suggest that, despite relatively high circulating endostatin levels, changes in vector design resulting in increased transgene expression levels and prolongation of circulating half-life of endostatin by fusion to a Fc portion of an immunoglobulin molecule may lead to improved anti-tumor activity of endostatin gene transfer. We thus will focus now on the improvement of the efficacy of this already promising antiangiogenic approach for breast cancer treatment.

As reported above, we put the construction of the gutless ADV on hold due to problems with helper virus contamination secondary to insufficient expression of Cre recombinase of the packaging cell lines. Instead of concentrating on vector design, our efforts were rather focused to prove the efficacy of endostatin gene transfer for breast cancer. To this end, we constructed an E1 minus recombinant adenovirus expressing endostatin under the control of the human EF-1α promoter, which resulted in a significant delay in tumor growth and total prevention of metastases formation [3]. Regression of pre-established tumors, however, could not be documented with this approach. It has been shown that different doses of endostatin are needed for the successful treatment of primary tumors or metastases [4]. In addition, we have shown that the effect of endostatin on the maturation of tumor vasculature is also dose dependent. There is an inverse relationship between endostatin serum levels and tumor vessel maturation [5]. Furthermore, local endostatin concentration was also shown to be important for the anti-tumor effect [6]. Therefore, the limited efficacy with the gene transfer approach observed in our study could be due to insufficient circulating endostatin levels or secondary to inefficient concentration of endostatin at the tumor site. We thus took a three-fold approach to improve the efficacy of endostatin gene transfer:

A. Changes in vector design in order to increase transgene expression levels and prolongation of circulating half-life of endostatin. Construction of an E1 minus adenovirus expressing a fusion protein between the Fc portion (hinge-CH2-CH3) of a murine immunoglobulin molecule (IgG2a) and endostatin under the control of the human EF-1α promoter.

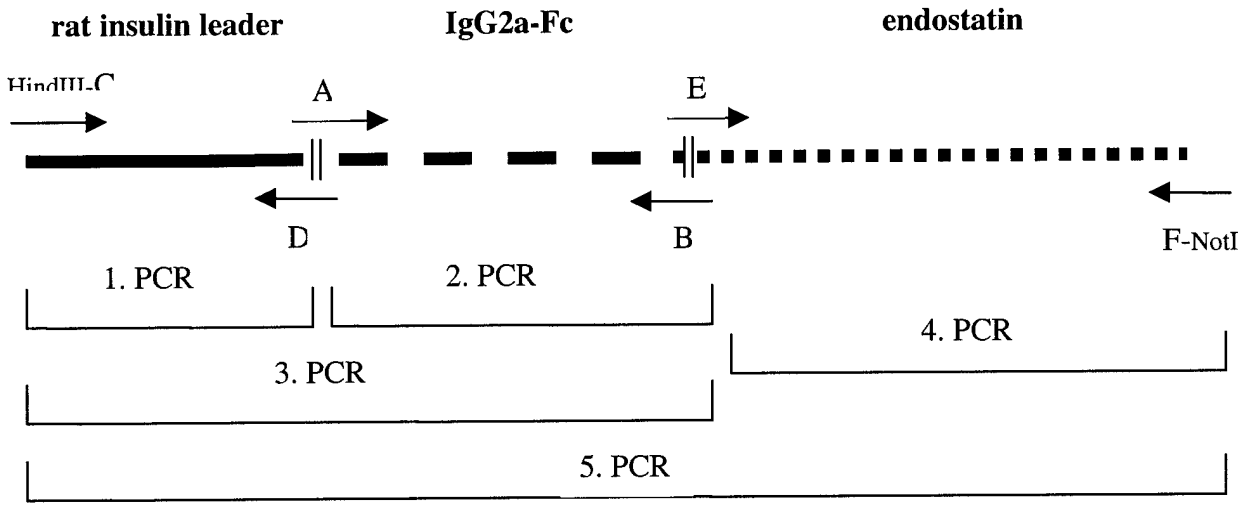
B. Exploration of potential mechanisms to target endostatin directly to the tumor site.

C. Transcriptional targeting of conditionally replicating adenoviruses (CRAD's) to activated endothelium.

A. Construction of ADV-Ig-end by overlapping PCR:

1. PCR: Total RNA was isolated from 1x10^7 splenocytes (RN'easy kit, Qiagen) and the hinge-CH2-CH3 portion of IgG2a was amplified using template specific primers (One-step RT-PCR, Qiagen): Primer A (sense) with 11 additional nucleotides on the 5' side coding for the 3' end of rat insulin leader sequence (CTGCCCAGGCTGAGCCCAGAGGGCCCACAAT) and primer B (antisense) with additional 11 nucleotides on the 3' end coding for the beginning of the endostatin gene (TGATGAGTATGTTTACCCGGAGTCCGG). A polymerase with proof-reading activity was used in all DNA amplification steps (pfu, Stratagene).
2. PCR: Primer C (sense): 5' end of rat insuling leader including Hind III restriction site and Kozak sequence (GAATTCAAGCTTGCCACCATGGCCCTGTGGA). Primer D (antisense): 3' end of rat insuling leader sequence plus 11 additional nucleotides of IgG2a Fc (CCTCTGGGCTCAGCCTGGGCAGGCTTGGGCTC).
3. PCR: Products from PCR 1 and 2 were gel purified, mixed and amplified with primers B and C to generate rat insulin leader – IgG2a .
4. PCR: The previously cloned endostatin cDNA was amplified with primer E (sense) containing on the 5' end 13 additional nucleotides of the 3' end of IgG2a cDNA. (GACTCCGGGTAAACATACTCATCAGGACTTTC) and primer F (antisense) including a Not I restriction site (GAAGAGTAAGCGGCCGCCTATTTGGAGAAA).
5. PCR: The so amplified endostatin cDNA served together with the rat insulin leader – IgG2a PCR product from the 3. PCR as a template for the last PCR using primer C (sense) and primer F (antisense): 3' end of endostatin including a Not I restriction site. The final PCR product was gel purified, cloned into the Hind III and Not I sites of the previously described adenovirus shuttle vector and sequenced.

Summary of IG-End PCR:



Recombinant E1 minus adenovirus was rescued using calcium phosphate co-transfection of E1 expressing 293 cells with the Ig-end adenovirus shuttle vector and an adenovirus backbone vector (pBHG10, Microbix) followed by agarose overlay. The resulting viral plaques (17) were harvested after 4 weeks. Viral DNA was extracted from the cell lysate of half of each plaque and digested with Hind III to verify the presence of the transgene. From the other half of each plaque, the virus was released from the cells by three freeze/thaw cycles and used for further virus amplification on 293 cells. The plaques were also screened for transgene expression by determining endostatin levels in plaque supernatants using an ELISA for murine endostatin (Cytimmune). Only one of the 17 plaques expressed the transgene (endostatin) at high levels (Fig. 1).

Fig. 1:

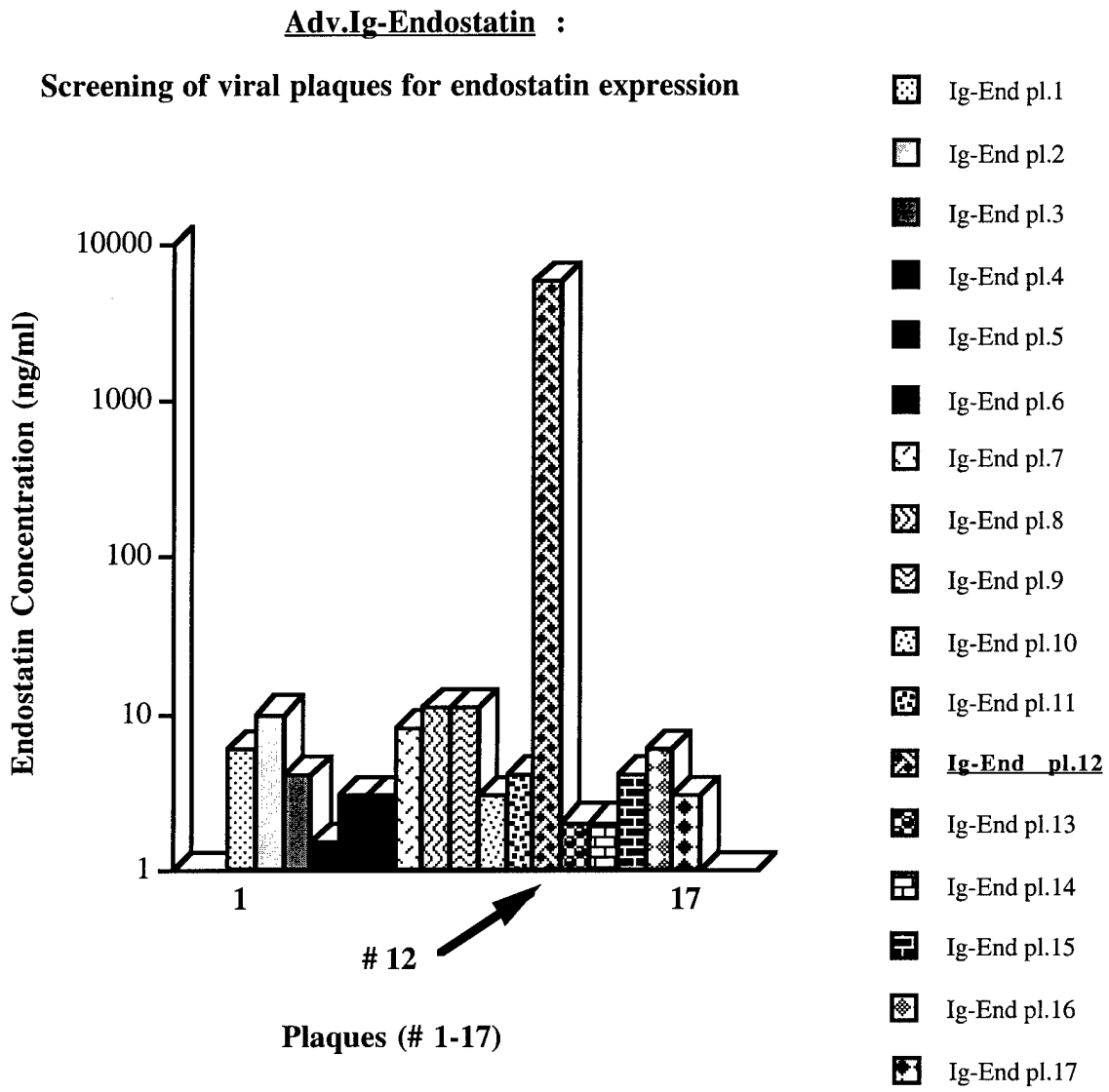


Fig.1: Endostatin expression in supernatant of viral plaques (ELISA). Only plaque number 12 is expressing endostatin at very high levels (~9000 ng/ml).

Plaque number 12 was then used for large scale virus preparation on 293 cells (500x 15 cm dishes). Virus was purified on two sequential CsCl ultracentrifugation steps. The final preparation was negative for both endotoxin and mycoplasm contamination. Viral particles (vp) as measured by OD_{260} were 2.85x10^12 vp/ml. Infectious titer (plaque forming units; pfu), determined by standard agarose overlay plaque assay on 293 cells, was 4.5x10^10 pfu/ml.

The purified Adv.Ig-end was tested side by side with the previously described Adv.End for transgene expression *in vitro*. Murine breast cancer cells (JC) were infected at different multiplicity of infection (moi) with the two endostatin viruses or control virus (Adv.LacZ) and endostatin levels were measured in the supernatant after 48 hours (Fig. 2).

Fig. 2:

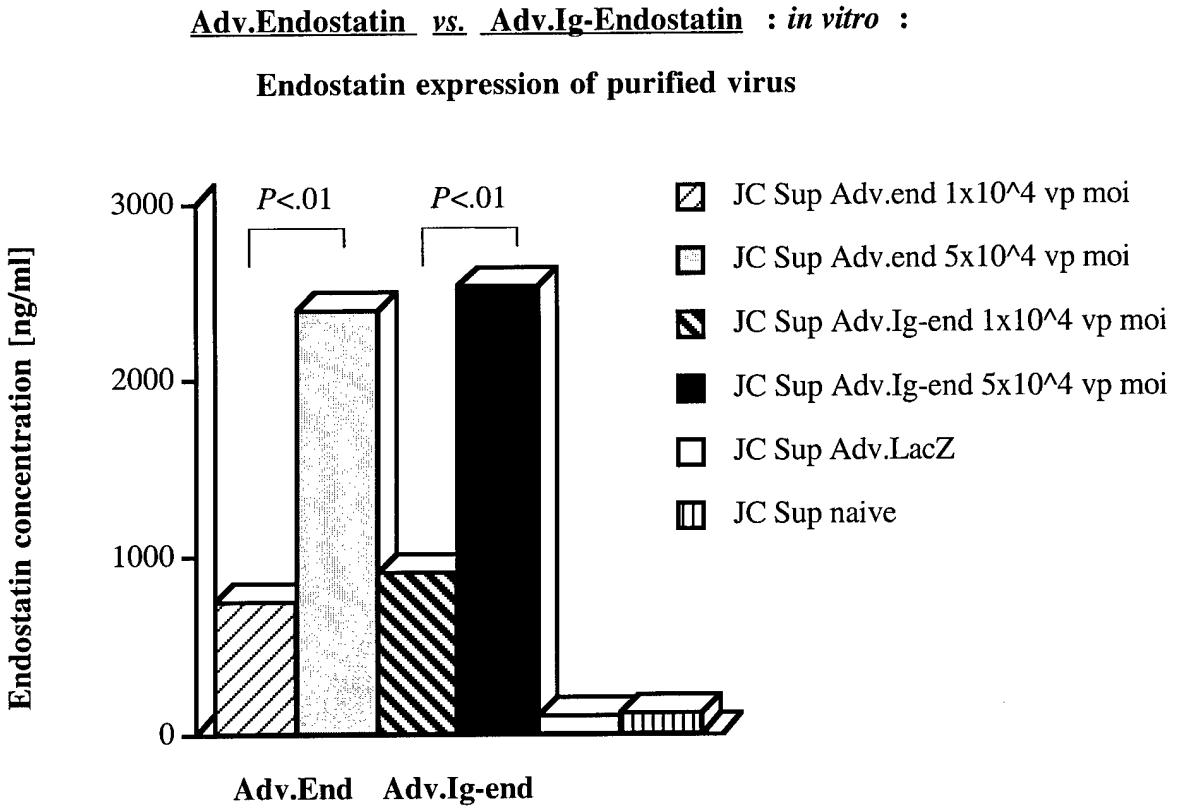


Fig. 2: Endostatin ELISA in supernatant of JC cells 48 hours after virus infection: Dose response of Adv.End (left two bars), and of Adv.Ig-end (middle two bars). Negative controls (Adv.LacZ and supernatant of non-infected JC cells) right two bars.

With both endostatin viruses, increasing viral dose resulted in significantly more endostatin production as detected in the supernatant of infected JC cells. Importantly, there was no difference in endostatin production with either viral dose when Adv.End was compared with Adv.Ig-end. The fusion protein was as effectively secreted as endostatin alone.

We then went ahead to compare endostatin expression levels *in vivo* (nude mice) after systemic administration (tail vein injection) of 1x10^11 vp of Adv.End or Adv.Ig-end. Endostatin serum levels were measured at 1 week, 3 weeks and 6 weeks after viral injection. There was no significant difference in expression levels at the three time points. The data of the 6 week time point are shown in Fig. 3.

Fig. 3:

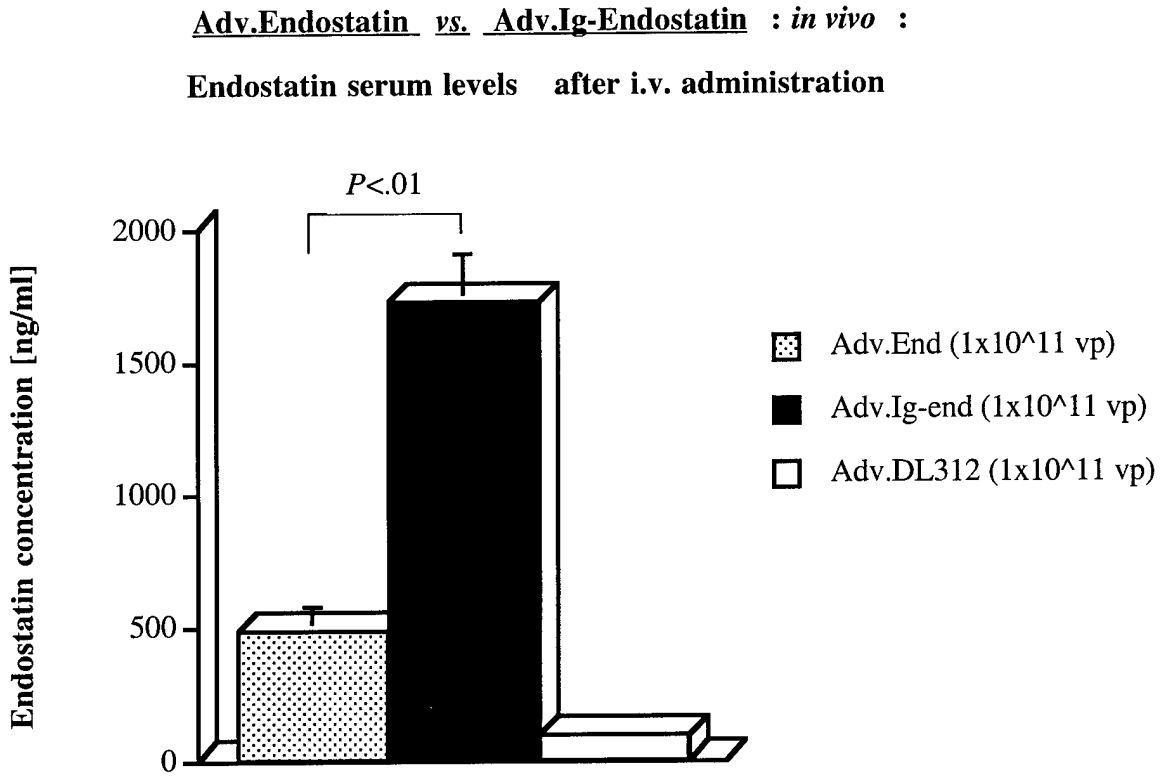


Fig. 3: Endostatin serum levels measured by ELISA six weeks after i.v. injection of 1x10^11 vp of Adv.End, Adv.Ig-end or control vector (Adv.DL312).

The endostatin levels after the injection of Adv.Ig-end were significantly higher at all time points than those with Adv.End.
These two endostatin viruses were tested *in vivo* head to head in preestablished murine breast cancers (JC). The tumors in the Adv.Ig-end group were smaller than in the Adv.-end group. The results, however, did not reach statistical significance. This maybe due to the size of the groups (N=7) (Fig. 4).

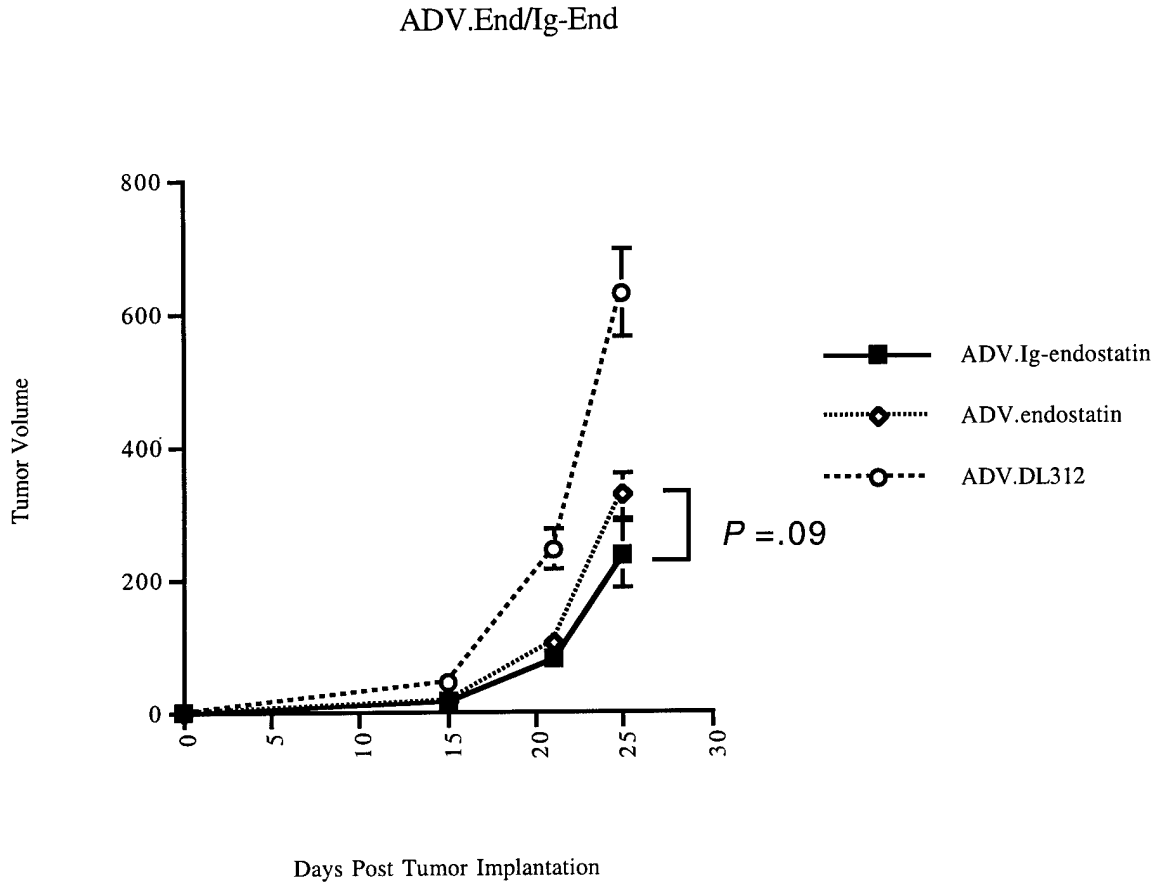


Fig. 4: JC tumors (breast cancer) after i.v. injection of 1x10^11 vp of Adv.End, Adv.Ig-end or control vector (Adv.DL312).

B. Targeting of endostatin to the tumor site:

It has become clear that local endostatin concentrations may play an important role in tumor control [6]. To explore the natural homing of endostatin to the tumor, we injected 1x10^11 vp of Adv.end or control vector (Adc.DL312) into tumor bearing mice. The mice were sacrificed after one week and tumors were stained immunohistochmically for endostatin. There was no significant difference in endostatin stainings between treated and control tumors.
We therefore wanted to explore if high local concentration of endostatin inhibits endothelial cell proliferation and induces apoptosis in growing endothelium. To this end, we induced an endothelial denudating injury by 3 passages of a 0.25-mm angioplasty guide wire (Advanced Cardiovascular Systems) through a mouse femoral artery one day after i.v. injection of 1x10^11 vp of Adv.End or Adv.DL312. This model has previously been described in detail [7]. Immunostaining for endostatin showed a massive accumulation of endostatin at the site of injury, but not at the contralateral non-injured control leg or after injection of control vector (Fig.5).

Fig. 5:

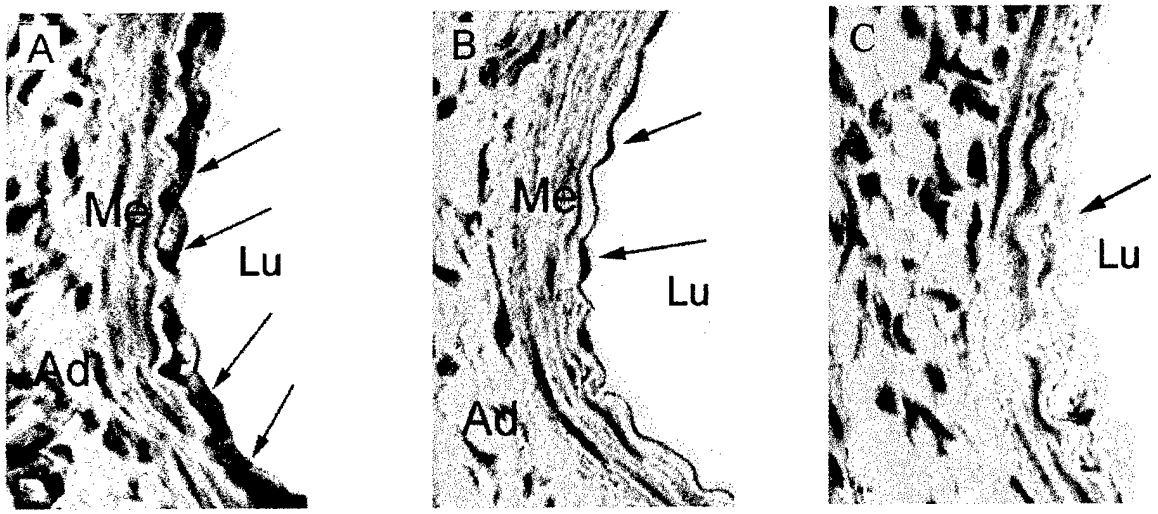


Fig. 5: Immunohistochemical staining of endostatin protein at the femoral arterial wall 1 week and 4 weeks following arterial injury: 1 week: (A) Injured, endostatin treated: A clearly

devoid of luminal endothelial coverage, indicates the strong presence of endostatin protein. (B) Non-injured, endostatin treated: Only weak staining of elastic fibers in the media of the non-injured artery can be seen and no endostatin protein is detectable at the luminal surface, which is completely covered by endothelial cells (arrows). (C) Injured, control vector treated: Very little endostatin staining along the denuded luminal surface (arrow).

Removal of endothelium allowed for local concentration of endostatin at the exposed basement membrane at the site of injury. To test for the biological effect of endostatin after the endothelial injury, quantitative computer assisted morphometry was used to measure the degree of re-endothelialization after endostatin gene transfer. Endostatin inhibited re-endothelialization of the denudation injury significantly at two and four weeks after injury when compared with control vector (Adv.DL312). Also, significantly more endothelial cell apoptosis was found in endostatin treated animals *vs.* controls as measured by activated caspase-3 expression (Fig. 6).

Fig. 6:

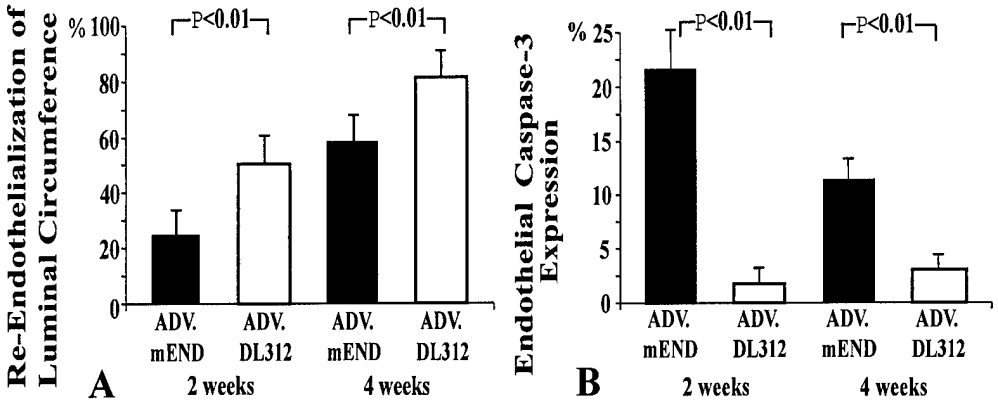


Fig. 6: Comparison of the effects of endostatin overexpression on re-endothelialization and luminal endothelial cell apoptosis in treatment (Adv.End: filled bars) and control (Adv.DL312: open bars) groups at 2 and 4 weeks after arterial injury and adenoviral injection. (A) Difference between percentage of the luminal circumference of the artery covered by endothelium (independent sample t-test $P < .01$) (B) Proportion of caspase-3 expressing endothelial cells in the luminal endothelium (independent sample t-test $P < .01$).

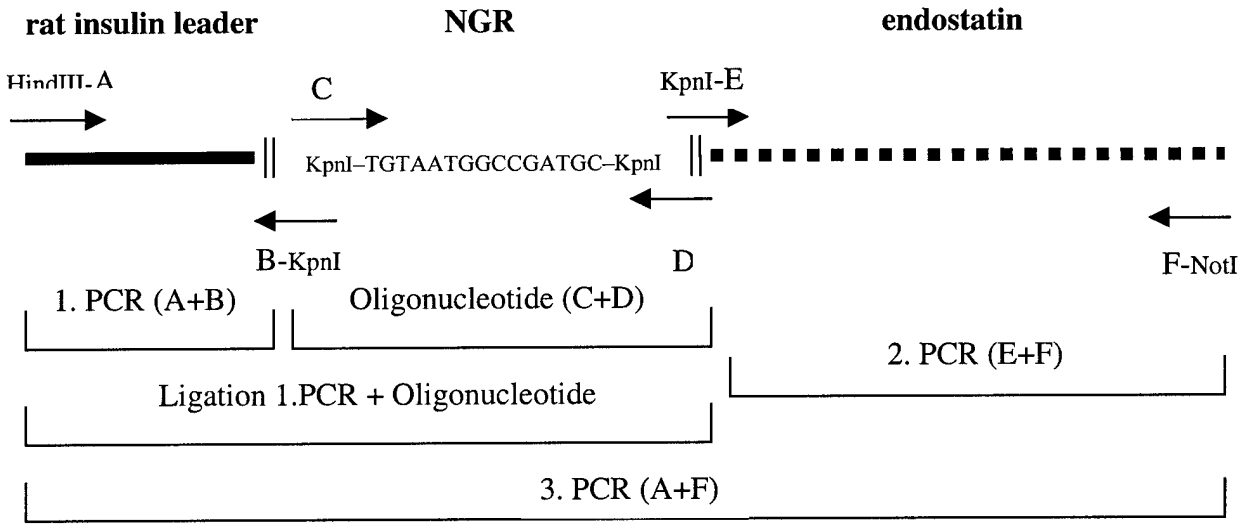
In summary, accumulated endostatin at the site of endothelial injury effectively induces apoptosis of the re-growing endothelium and inhibits proliferation of activated endothelial cells.

Having shown in this model that locally deposited endostatin exerted such dramatic effects on endothelial survival at the site of endothelial turnover we sought to apply this principle to anti-angiogenic cancer therapy.

Recently, utilizing phage display techniques, several short peptides (NGR, NG2, etc.) have been identified that specifically home to tumor endothelium/activated endothelial cells [8]. By fusing these peptide sequences to endostatin we intended to "home" endostatin directly to the site of the tumors.
Employing overlapping PCR techniques as described above we made two different "endothelium-targeted" endostatin constructs:

1. NGR-endostatin
2. RGD-endostatin

Summary of NGR-End Construction:



The RGD-endostatin was constructed by replacing the NGR peptide sequence between the two KpnI sites with a RGD motive.

The recombinant adenovirus expressing these sequences has just been successfully rescued. The sequence of the transgenes corresponded to the above constructs.
These two viruses will now be tested *in vitro* for expression and *in vivo* regarding the tumor targeting of the circulating transgene as well as anti-tumor efficacy.

C. Transcriptional targeting of Conditionally Replicating Adenovirus (CRAD) to dividing endothelial cells:

We constructed CRADs targeted to dividing endothelial cells, which are present in the tumor endothelium. We utilized the regulatory elements of Flk-1 and endoglin genes, which have been shown to be highly overexpressed in angiogenic endothelial cells, to construct two CRADs: Ad.Flk-1, which has adenoviral E1A gene under the control of the Flk-1 enhancer/promoter, and Ad.Flk-Endo, which harbors the same Flk-1 enhancer/promoter as Ad.Flk-1, plus it has the adenoviral E1B gene under control of the endoglin promoter. Using viral replication assays we showed that these viruses replicate with high selectivity only in endothelial cells and not in other cell lines. Moreover, in 3-D angiogenesis matrigel assays the CRAD's effectively destroyed the budding capillary networks as opposed to the control viruses. These results are reported in detail in a forthcoming publication [9], which is currently *in press* in the journal *Gene Therapy*. A complete manuscript is included in the appendix.
This endothelial-targeted CRAD will now be used to express anti-angiogenic genes, such as endostatin.

Key Research Accomplishments

- Construction of a recombinant adenovirus expressing high levels of murine endostatin
- Demonstration of its anti-tumor effect and the prevention of metastasis formation
- Construction of a recombinant adenovirus expressing a fusion protein between the Fc portion of murine IgG2a and murine endostatin (Adv.Ig-end)
- Demonstration of equally effective secretion *in vitro* from virus infected cells of the Ig-endostatin fusion protein when compared to endostatin alone
- Demonstration of significantly increased endostatin serum levels and better *in vivo* effect after systemic administration of Adv.Ig-end compared to Adv.End
- Demonstration of the potent biological activity of endostatin (inhibition of endothelial cell growth; induction of endothelial cell apoptosis) after local accumulation of endostatin at the site of endothelial repair
- Construction of an endostatin molecule with endothelium homing/targeting
- Construction of conditionally replicating adenovirus (CRAD) targeted to the activated endothelium

Reportable Outcomes

1. B. Sauter, O. Martinet, W.-J. Zhang, J. Mandeli, and S. L.C. Woo. Adenovirus-mediated gene transfer of endostatin in vivo results in high level of transgene expression and inhibition of tumor growth and metastases. *Proc Natl Acad Sci* 2000; 97: 4802-4807

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3. R. Hutter, E. Reis, S. L.C. Woo, and B. Sauter. Endostatin gene transfer inhibits tumor vessel maturation proportionally to transgene expression levels. *Circulation.* Poster presentation AHA (American Heart Association) Meeting 11/2000.

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Conclusions

Our data on adenovirus-mediated gene transfer of endostatin has clearly shown the efficacy of an antiangiogenic gene therapy approach for cancer: Significant delay in tumor progression, and more importantly, complete prevention of lung metastases formation. Regression of pre-established tumors, however, has not been achieved, which maybe due to insufficient circulating endostatin levels or secondary to low local concentration of endostatin at the tumor site.

Construction of a recombinant adenovirus expressing an Ig-endostatin fusion protein resulted in significantly elevated serum endostatin levels *in vivo*. This construct showed an increased anti-angiogenic activity in a murine breast cancer model.

Furthermore, local endostatin concentration was also shown to be important for the anti-tumor effect [6]. Thus, a specifically tumor-targeted endostatin construct was made using peptides that home selectively to activated endothelium to increase the efficacy of endostatin gene transfer. These constructs are being tested now.

In addition, a conditionally replicating adenovirus (CRAD) that replicates selectively in activated endothelium was developed. This virus will now be used to deliver anti-angiogenic genes directly to the endothelium.

Finally, the most potent anti-angiogenic construct of the above tested ones will be used in combination with immunomodulatory tumor therapy or even conventional anti-cancer treatments. This is likely to have additive or even synergistic effects as it was already shown with other angiogenesis inhibitors [10].

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Appendices

1. Papers/Abstracts:

2. CV

Adenovirus-mediated gene transfer of endostatin *in vivo* results in high level of transgene expression and inhibition of tumor growth and metastases

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Communicated by M. Judah Folkman, Harvard Medical School, Boston, MA, February 15, 2000 (received for review August 10, 1999)

Inhibition of angiogenesis has been shown to be an effective strategy in cancer therapy in mice. However, its widespread application has been hampered by difficulties in the large-scale production of the antiangiogenic proteins. This limitation may be resolved by *in vivo* delivery and expression of the antiangiogenic genes. We have constructed a recombinant adenovirus that expresses murine endostatin that is biologically active both *in vitro*, as determined in endothelial cell proliferation assays, and *in vivo*, by suppression of angiogenesis induced by vascular endothelial growth factor 165. Persistent high serum levels of endostatin (605–1740 ng/ml; mean, 936 ng/ml) were achieved after systemic administration of the vector to nude mice, which resulted in significant reduction of the growth rates and the volumes of JC breast carcinoma and Lewis lung carcinoma ($P < 0.001$ and $P < 0.05$, respectively). In addition, the endostatin vector treatment completely prevented the formation of pulmonary micrometastases in Lewis lung carcinoma ($P = 0.0001$). Immunohistochemical staining of the tumors demonstrated a decreased number of blood vessels in the treatment group versus the controls. In conclusion, the present study clearly demonstrates the potential of vector-mediated antiangiogenic gene therapy as a component in cancer therapy.

antiangiogenesis | cancer | gene therapy

In recent years it has become clear that angiogenesis not only is important in physiological processes such as embryonic development, wound healing, and organ and tissue regeneration, but also plays a pivotal role in tumor progression and metastasis (1). The target of antiangiogenic cancer treatment is the genetically normal endothelial cell. Therefore, the development of resistance to angiostatic therapy is very unlikely and has not been reported so far (2). If a cancer exceeds the size of ≈1–2 mm, recruitment of new blood vessels is needed (angiogenesis) to prevent tumor cell apoptosis. Tumor cells promote angiogenesis by the secretion of angiogenic factors, in particular basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (3). Recently, evidence emerged that angiogenesis is tightly regulated by a balance of activating and inhibiting factors (4). Therefore, continuous overexpression of antiangiogenic factors by gene therapy, for instance, should counteract the tumor-induced angiogenesis.

Many tumor- and non-tumor-associated antiangiogenic factors have been described. The proteolytic cleavage of larger precursor molecules associated with the vascular system (proteins of the coagulation cascade and basement membrane proteins) is thought to play an important role in the generation of several of these antiangiogenic proteins and, thus, in the control of angiogenesis. Of the known angiogenesis inhibitors, the recently discovered protein endostatin, a 20-kDa (184 aa) C-terminal fragment of collagen XVIII, is the most potent inhibitor of tumor angiogenesis described so far (5).

Despite intensive efforts, large quantities of recombinant protein sufficient for clinical trials were not available until recently (6). The difficulties in protein production, long-term storage of bioactive protein, and the cumbersome daily administration may be overcome through transfer of the endostatin gene. Two recent reports using nonviral gene therapy with endostatin show some efficacy against tumor growth (7, 8). The systemic endostatin levels achieved in these studies, however, were rather low. Because there is a dose–response relationship between endostatin concentration and its antiangiogenic effect in all reported *in vitro* and *in vivo* studies, we chose the highly efficient adenovirus-based gene delivery system for our model to maximize transgene expression. Successful antiangiogenic gene therapy with adenoviral vectors already has been shown by several other groups (9–12).

In this paper, we demonstrate the ability to generate persistent high levels of circulating endostatin levels through adenovirus-mediated gene therapy, its effect on tumor growth in two different tumor models [a murine breast cancer cell line (JC) and Lewis lung carcinoma (LLC)], and, more importantly, the complete prevention of lung metastases formation in LLC.

Materials and Methods

Murine Endostatin cDNA Cloning and Adenovirus Construction. Liver tissue from a BALB/c mouse was homogenized, and total RNA was extracted (RNeasy kit; Qiagen, Chatsworth, CA). First-strand cDNA was amplified by reverse transcription–PCR with oligo(dT) primers (SuperScript II; Life Technologies, Grand Island, NY). The full-length mouse endostatin cDNA was amplified by PCR (sense primer with a *Cla*I linker, ATCGATCATACTCATCAGGACTTTCAGCC; antisense primer with a *Not*I linker, GCGGCCGCCTATTTGGAGAAAGAGGTCAT) for subcloning into pBluescript (Stratagene). A synthetic oligonucleotide coding for the rat insulin leader sequence was cloned in front of the endostatin gene. After sequence confirmation, the rat insulin leader endostatin cDNA was cloned into the recombinant adenovirus (ADV) shuttle vector pADV.hEF1-α (human elongation factor 1-α) for the rescue of the recombinant adenovirus as described (13). The viral particles were measured by absorption (A_{260}), and the plaque-forming units (pfu) were determined by standard agarose-overlay plaque assay on 293 cells.

Abbreviations: ADV, recombinant adenovirus; ATTV, average total tumor volume; bFGF, basic fibroblast growth factor; E1, adenovirus early gene 1; GST, glutathione *S*-transferase; HUVEC, human umbilical vein endothelial cells; LLC, Lewis lung carcinoma; moi, multiplicity of infection; pfu, plaque-forming units; VEGF, vascular endothelial growth factor; ICAM-1, intercellular adhesion molecule-1.

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Recombinant Adenoviruses. All recombinant adenoviruses used in this study were generated in our laboratory as described above. The cDNA for the construction of the ADV.hVEGF165 was obtained through reverse transcription–PCR of RNA isolated from human umbilical vein endothelial cells (HUVEC).

Cell Lines. JC and LLC cell lines were obtained from American Type Culture Collection. The cells were cultured in RPMI medium 1640 (JC) and DMEM (LLC). All media were supplemented with 10% FBS, 0.2 mM glutamine, and 1% penicillin/streptomycin. HUVEC were isolated from umbilical cords (Institutional Review Board-approved cord blood program) by collagenase type IV (Sigma) perfusion (0.2% in Hanks' balanced salt solution) for 20 min at room temperature. The cells then were cultured on collagen-coated (1% in PBS) plates in M199 medium supplemented with 20% FBS, 0.2 mM glutamine, 1% penicillin/streptomycin, and 1 ng/ml bFGF.

Generation of Purified Endostatin. Murine endostatin was cloned into a glutathione *S*-transferase (GST) fusion vector (pGEX-4T-1; Amersham Pharmacia) and expressed in *Escherichia coli* as a 46-kDa GST-endostatin fusion protein. After induction, >90% of the GST-endostatin fusion protein remained in bacterial inclusion bodies. The soluble GST-endostatin fraction was eluted from a 50% slurry of PBS/glutathione-Sepharose 4B (Amersham Pharmacia) with 10 mM reduced glutathione in 50 mM Tris·HCl, pH 8.0. Soluble recombinant endostatin was generated by infection of breast cancer cells with ADV.mEnd at a multiplicity of infection (moi) of 100 pfu. After 48 h, the serum-free supernatant was harvested and sequentially concentrated over Centriplus spin columns (molecular mass cut-off of 50,000 Da followed by 30,000 Da; Amicon). The purified protein was analyzed on a 10% reducing SDS/PAGE gel.

Production of Polyclonal Endostatin Antiserum. The inclusion bodies with GST-endostatin fusion protein were solubilized with 8 M urea and loaded on a preparative SDS/PAGE gel (Protean II; Bio-Rad). The correct-size band was excised and turned over to Research Genetics (Huntsville, AL) for immunization and for the production of polyclonal rabbit anti-endostatin antiserum.

Western Blot of ADV.mEnd Conditioned Supernatant. JC cells were transduced at a moi of 100 pfu with ADV.mEnd and control vector ADV.β-Gal. Conditioned supernatant was harvested after 48 h and concentrated as described above. Samples (5 μl) of the different fractions (50/30-kDa cut-off and flow-through) were separated on a 10% reducing SDS/PAGE gel and transferred to a poly(vinylidene difluoride) membrane (Hybond-P; Amersham Pharmacia). The membrane was probed with the polyclonal rabbit anti-endostatin antiserum, followed by a donkey anti-rabbit FITC-conjugated antibody and a signal amplification step (Vistra Fluorescence Western blotting kit; Amersham Pharmacia). The signals were analyzed with a Storm 860 PhosphorImager (Molecular Dynamics) and IMAGEQUANT software.

***In Vitro* Bioactivity Assay of Endostatin.** Proliferation inhibition assay using a 2-fold dilution series of conditioned supernatant (ADV.mEnd or ADV.β-Gal) on HUVEC was performed as previously described (5). HUVEC proliferation was measured by a tetrazolium-based assay (EZ4U kit; Biomedica, Vienna).

***In Vivo* Angiogenesis Assay.** Athymic nude mice, purchased from the National Cancer Institute, were s.c. injected with 1×10^{11} particles of ADV.hVEGF165 or the adenovirus early gene 1 (E1)-deleted adenovirus control vector ADV.DL312 into an India ink-marked area of the right and left lateral thigh, respectively. The mice were divided into three groups and injected via tail vein with (*i*) PBS, (*ii*) 1×10^{11} ADV.DL312 particles, and (*iii*) 1×10^{11} ADV.mEnd particles. At day 14, the animals were killed, and the marked injection sites on the right and left thighs (ADV.hVEGF165 and ADV.DL312, respectively) were subjected to histological analysis with hematoxylin/eosin staining and immunohistochemistry.

VEGF and Endostatin Measurements. Endostatin levels in conditioned supernatant and serum and human VEGF serum levels were measured with commercially available ELISA kits (CytImmune Sciences, College Park, MD, and R & D Systems).

***In Vivo* Evaluation of Tumor Growth.** Cells (1×10^5 LLC or JC) were inoculated s.c. into nude mice. Tumors were measured with calipers in two dimensions every 4 to 5 days, and the volume was calculated as length $\times$ width$^2 \times 0.52$.

Immunohistochemistry. Sections were probed with a monoclonal rat anti-mouse intercellular adhesion molecule-1 (ICAM-1) antibody (1:300; Seikagaku America, Rockville, MD), followed by a biotinylated polyclonal rabbit anti-rat antibody (1:100; Vector Laboratories).

Statistical Analyses. For comparison of individual time points, ANOVA and unpaired Student's *t* tests were used. For each animal, the average total tumor volume (ATTV) was calculated from the first tumor measurement to the end of the experiment. The ATTV equals the area under the curve (AUC) of the tumor volumes over time divided by the number of days [e.g., ATTV = AUC/number of days = AUC/(26 − 7) = AUC/19]. The ATTV represents the average height of the volume–time curve from day 6 to day 27, thereby giving an overall index of tumor size. *P* values were calculated by using the Wilcoxon Rank Sum Test (14).

Results

Construction of a Recombinant Adenovirus Expressing Murine Endostatin. The cDNA of murine endostatin was PCR-cloned from a mouse liver, and a synthetic oligonucleotide coding for the rat insulin leader sequence was added at the 5′ end. The recombinant replication-deficient adenovirus was generated through cotransfection of the expression cassette containing an adenovirus shuttle vector with an E1-deleted adenovirus backbone vector (pJM-17) (Fig. 1*A*). A restriction digestion of viral DNA with *Hin*dIII was performed to verify the rearrangement of restriction fragments of ADV.mEnd DNA compared with the backbone adenoviral vector plasmid, confirming the correct insertion of the transgene. Aside from minor band shifts, the loss of the 3,437-bp DNA fragment in the recombinant virus as compared with the plasmid is the hallmark of transgene insertion into the E1 region of the adenovirus (Fig. 1*B*). A murine breast cancer cell line with a high transduction efficiency by adenovirus was transduced with an moi of 100 pfu of ADV.mEnd or ADV.β-Gal, respectively, and serum-free supernatant was sequentially concentrated over two columns with a molecular mass cut-off of 50 kDa followed by 30 kDa. Then, 5 μl of the concentrated supernatant was separated on a reducing 10% SDS/PAGE gel, and a distinct band at around 22 kDa was visualized in the ADV.mEnd conditioned supernatant but not in the control. The 22-kDa band, corresponding to the size of endostatin, was seen in both the 50-kDa and the 30-kDa cut-off fractions but not in the flow-through of the 30-kDa column, indicating that protein charges and conformation influenced its filtration through the membranes. Incomplete reduction of multimers and association with other cellular proteins in this highly overexpressed system may have accounted for the retention in the 50-kDa cut-off column (Fig. 1*C*). Polyclonal rabbit anti-endostatin antibody, raised against a GST-endostatin fusion

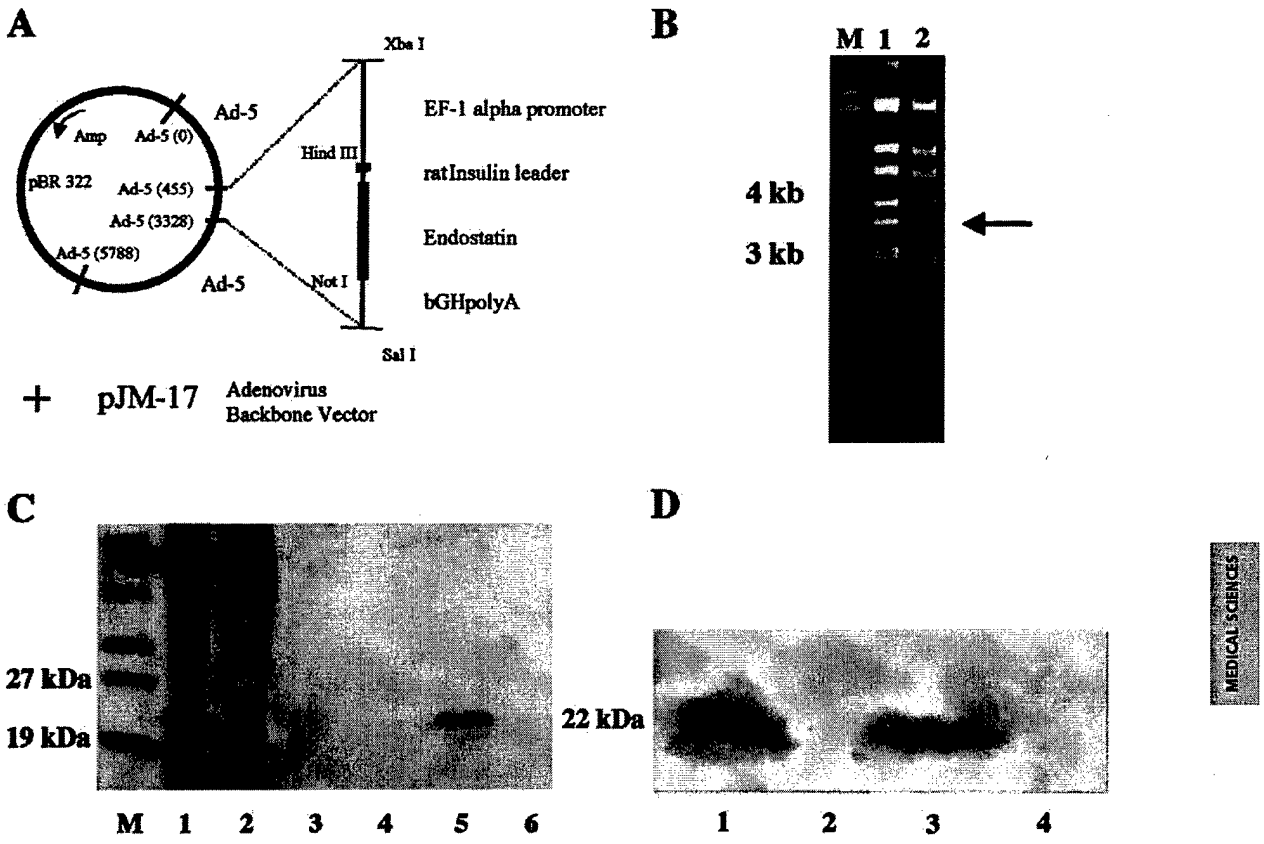

Fig. 1. Construction, molecular analysis, and transgene expression of ADV.mEnd. (*A*) The expression cassette consisting of human elongation factor-1 α (EF-1α) promoter, the rat insulin leader sequence, the cDNA of murine endostatin, and the bovine growth hormone poly(A) (bGHPA) was cloned in an adenovirus shuttle vector. Recombinant adenovirus was generated on cotransfection of the shuttle plasmid with the adenovirus backbone plasmid pJM-17. (*B*) Rearrangement of *Hind*III restriction fragments (loss of a 3,437-bp fragment (arrow), emergence of a 1,003-bp fragment (data not shown), and other minor band shifts of the adenovirus backbone plasmid (lane 1) as compared with the recombinant ADV.mEnd (lane 2) confirmed the correct insertion of the transgene into the E1 region in the adenovirus. (*C*) Ten percent reducing SDS/PAGE of serum-free concentrated supernatant of virally transduced breast cancer cells. Retention on 50-kDa molecular mass cut-off column, ADV.mEnd (lane 1) and ADV.β-Gal (lane 2); flow-through of 30-kDa cut-off column, ADV.mEnd (lane 3) and ADV.β-Gal (lane 4); retention on 30-kDa molecular mass cut-off column, ADV.mEnd (lane 5) and ADV.β-Gal (lane 6). (*D*) Western blot of the same supernatant as shown in *C*. Lanes 1 and 2 are the same as in *C*, and lanes 3 and 4 correspond to lanes 5 and 6 in *C*. M, molecular mass markers.

protein, reacted positively in a Western blot with the 22-kDa protein only. Preimmune serum in all fractions and immune serum against conditioned medium of the control vector did not generate any signals, thereby confirming both the specificity of the anti-endostatin antibody and that endostatin is efficiently secreted into the supernatant of ADV.mEnd-transduced cells (Fig. 1*D*).

ADV.mEnd-Expressed Endostatin Is Biologically Active *in Vitro* and *in Vivo*. To evaluate the biological function of the recombinant endostatin, we tested the purified protein from serum-free concentrated supernatant of adenovirally transduced cells in a proliferation inhibition assay on HUVEC. A dose–response between endostatin concentration and percentage inhibition of bFGF-stimulated HUVEC proliferation could be demonstrated (Fig. 2*A*). The ED_{50} of endostatin (975 ng/ml) was in the range of previously published data (15), but was a little higher than in the original paper (5). Similarly, nonconcentrated conditioned supernatant of JC cells transduced with ADV.mEnd strongly inhibited HUVEC proliferation with an ED_{50} at a dilution of 1:16, whereas ADV.β-Gal conditioned supernatant did not inhibit proliferation (Fig. 2*B*). As measured by a commercially available ELISA, endostatin concentration in the conditioned supernatant ranged from 12 to 16 μg/ml. There was no effect of purified endostatin or conditioned supernatant on the growth of JC cells (data not shown).

In vivo antiangiogenic activity of adenovirally expressed endostatin was tested by s.c. injection of 1×10^{11} particles of ADV.hVEGF165 or ADV.DL312 into India ink-marked areas of the right and left lateral thighs of a nude mouse, respectively. Particles (1×10^{11}) of ADV.mEnd or ADV.DL312, or PBS was then administered systemically through the tail vein. The left ADV.DL312-injected thigh served as an internal control in each mouse for potential local, nonspecific adenoviral effects. Mice were killed 14 days after viral injection, and the marked area was examined histologically. Histological sections were stained with hematoxylin/eosin and for ICAM-1. There was a clear inhibition of VEGF165-induced angiogenesis in the ADV.mEnd-treated mice vs. the mice injected with the control vector (Fig. 3). ADV.mEnd treatment resulted in endostatin levels from 625 to 1612 ng/ml. There was no increase in systemic angiogenesis as evidenced by the lack of detectable serum levels of human VEGF and by the absence of increased angiogenesis in the contralateral ADV.DL312-injected thigh (data not shown).

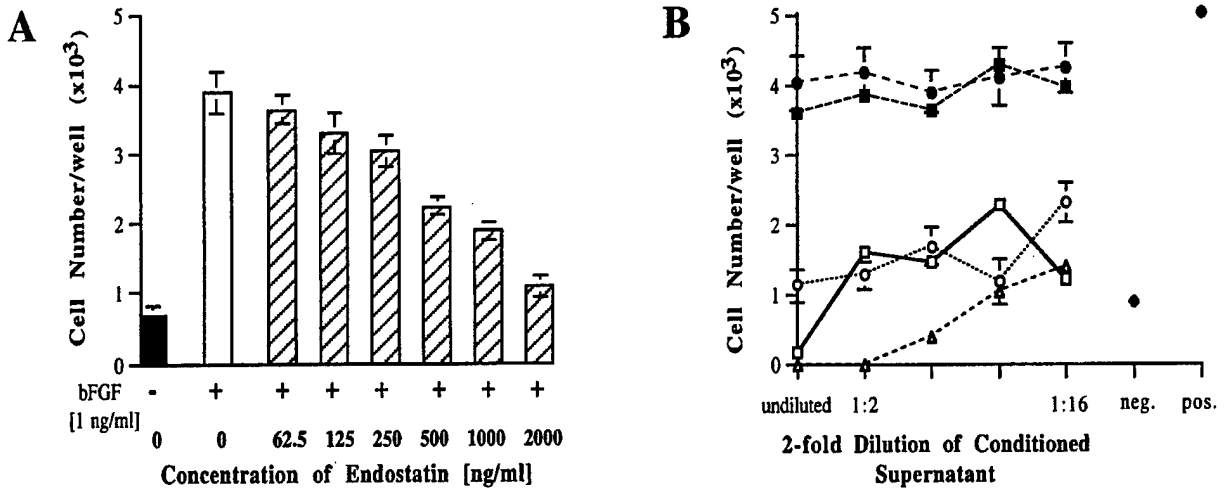

Fig. 2. *In vitro* assay for endostatin activity. (*A*) Inhibition of bFGF (1 ng/ml) stimulated HUVEC proliferation by increasing amounts of purified, ADV.mEnd-expressed endostatin (hatched columns) and in the absence of endostatin (positive control; open column). HUVEC proliferation without bFGF (negative control; filled column). (*B*) Same assay as in *A*. Shown is a 2-fold dilution of conditioned supernatant of JC cells transduced with ADV.mEnd or ADV.β-Gal at different moi (in particles). ADV.mEnd: 1×10^4 (○), 5×10^4 (□), and 1×10^5 (△). ADV.β-Gal: 1×10^4 (●) and 5×10^4 (■). pos., Complete medium with bFGF; neg., complete medium without bFGF. Data are shown as the mean of six wells. Bars indicate ± SD.

Systemic ADV.mEnd Administration Significantly Delays Tumor Engraftment. In the first *in vivo* experiment, we asked whether systemic administration of ADV.mEnd could prevent the formation of s.c. breast cancer (JC) in nude mice. Particles (1×10^{11}) of ADV.mEnd or control vector (ADV.DL312) were injected i.v. into the tail vein, followed by s.c. tumor-cell implantation (1×10^5 cells) 2 days later (20 animals per group). Animals were checked daily, and the emerging tumors were measured twice a week. By day 12 after tumor implantation, a statistically significant difference could be observed between treated and control groups ($P = 0.014$), with four animals in each group not having developed a tumor. Eventually, all animals developed tumors, but the growth rate of the ADV.mEnd-treated group was significantly slower than that in the controls, resulting in a 60% tumor size reduction in the treatment group at day 28 ($P = 0.0008$) (Fig. 4). Endostatin levels ranged from 810 to 1740 ng/ml in the treatment group and from 178 to 190 ng/ml in the control group.

ADV.mEnd Treatment Reduces Tumor Growth and Prevents Metastases. In a more clinically relevant situation, we treated preestablished tumors with ADV.mEnd. As a tumor model, we chose LLC for its general resistance to conventional tumor therapies, according to the National Cancer Institute screening panel for anticancer drugs. LLC cells (1×10^5) were inoculated s.c., and after establishment of a sizable tumor after 7 days (mean tumor volume, 47 mm³; range, 28–64 mm³), recombinant adenovirus was injected i.v. by tail vein (in nine treatment and seven control animals). A significant tumor reduction in the treatment group was already seen at day 6 after virus injection ($P = 0.048$). At day 26, when the control animals had to be killed for tumor burden, the decrease in tumor volume for the endostatin group reached

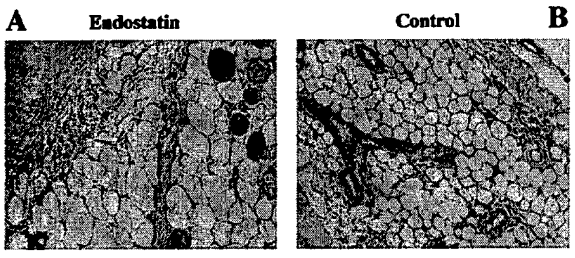

Fig. 3. *In vivo* assay for activity of ADV.mEnd-expressed endostatin. An adenovirus expressing hVEGF165 was injected s.c. into nude mice. At the same time, ADV.mEnd or control vector (ADV.DL312) was administered through the tail vein. After 14 days, the animals were killed, and the ADV.hVEGF165 injection site was histologically analyzed. ICAM-1 staining shows no s.c. blood vessels in the ADV.mEnd-treated animals (*A*) but showed increased angiogenesis in the ADV.DL312-treated animals (*B*). (×100.)

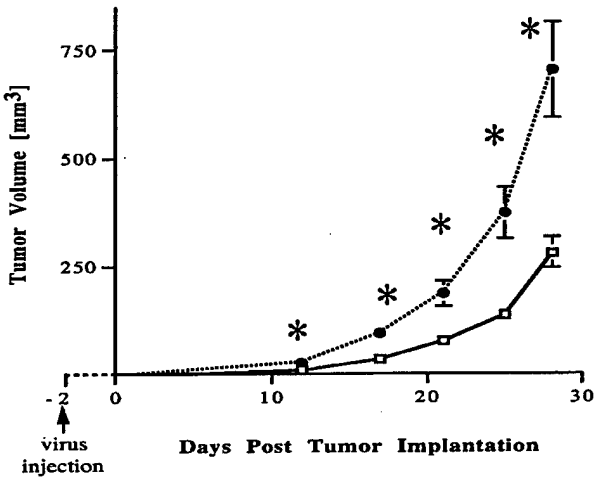

Fig. 4. Delay of tumor engrafting by ADV.mEnd. Recombinant adenovirus was injected i.v. 2 days before s.c. implantation of 1×10^5 JC cells. At 12 days after tumor implantation, a statistically significant difference in tumor size ($P = 0.01$) between the ADV.mEnd-treated (□) and the control vector-treated (ADV.DL312, ●) groups was observed. Tumor volume reduction at day 28 was 60% for the treatment group vs. controls ($P = 0.0008$). *, Statistically significant at the $P = 0.01$ level by unpaired Student's *t* test. Overall index of tumor size, median ATTV: ADV.mEnd, 88 mm³; ADV.DL312, 190 mm³. $P = 0.003$ (Wilcoxon rank sum test). There were 20 mice per group. Bars indicate ± SEM.

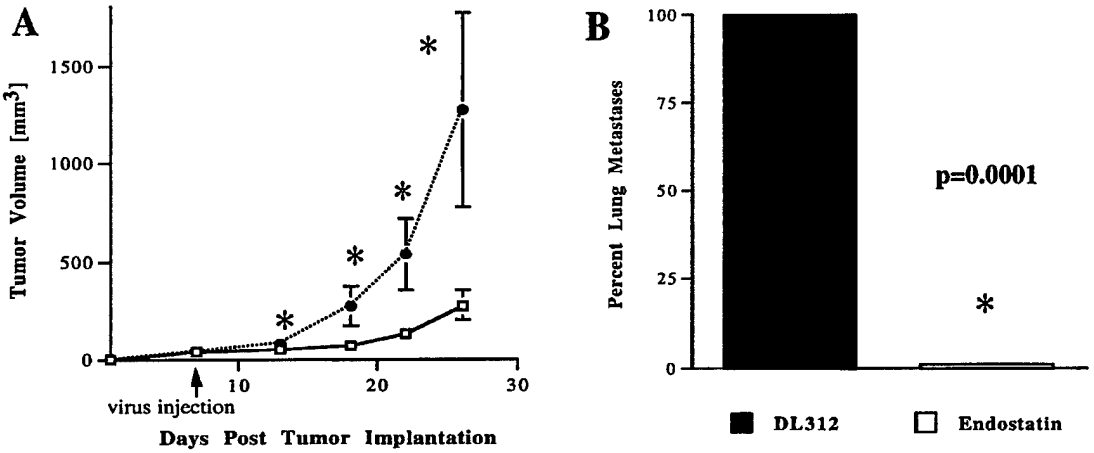

Fig. 5. ADV.mEnd treatment reduces tumor growth of preestablished tumors and prevents formation of lung metastases. LLC cells (1×10^5) were injected s.c. into nude mice. Virus treatment by tail vein injection was given at day 7 (mean total volume, 47 mm³). (*A*) At 6 days after virus injection, the tumors in the endostatin group (□; nine mice) were already significantly smaller than the controls (●; seven mice). At day 26, the total volume reduction was 78% for the treatment group vs. controls (P = 0.041). *, Statistically significant at the $P = 0.05$ level by unpaired Student's *t* test. Overall index of tumor size, median ATTV: ADV.mEnd, 96 mm³; ADV.DL312, 208 mm³. $P = 0.05$ (Wilcoxon rank sum test). (*B*) None of the ADV.mEnd-treated mice had lung micrometastases as opposed to 100% of the ADV.DL312-treated controls who did ($P = 0.0001$, Fisher's exact test).

78% ($P = 0.041$) (Fig. 5*A*). Moreover, histological analysis of the lungs of the LLC-bearing animals revealed that none of the nine ADV.mEnd-treated animals developed lung micrometastases, whereas all control animals were found to have tumor in the lungs ($P = 0.0001$; Fig. 5*B*). Endostatin serum levels were similar as in the above experiments with 605-1524 ng/ml after treatment with ADV.mEnd and 165–178 ng/ml in the control group.

Persistent High Serum Levels of Endostatin After a Single Injection of ADV.mEnd. Mean serum endostatin levels, including all of the tumor-bearing mice, were 936 ng/ml (range, 605-1740 ng/ml) at 26–28 days after injection of ADV.mEnd and 176 ng/ml (range, 165–190 ng/ml) for the ADV.DL312-treated groups ($P < 0.0001$) at the same time period.

ADV.mEnd Reduces Tumor-Induced Angiogenesis. Histological analysis by standard hematoxylin/eosin staining and immunohistochemistry with ICAM-1 of both the LLC and JC tumors showed decreased vascularization in the ADV.mEnd-treated animals (Fig. 6). A striking induction of angiogenesis was especially observed directly at the interface of the tumor and the surrounding s.c. tissue in the control vector-treated JC tumors (Fig. 6D_1).

Discussion

We have constructed a recombinant adenovirus expressing biologically active murine endostatin (ADV.mEnd) as shown in both *in vitro* and *in vivo* models of angiogenesis. Treatment of tumor-bearing nude mice with ADV.mEnd led to a tumor volume reduction of up to 78% as compared with the controls and, more importantly, completely prevented the formation of lung metastases in an LLC model. Histological data demonstrated decreased tumor vascularization and less angiogenesis around the tumor in the endostatin-treated animals. Difficulties in large-scale production of recombinant endostatin have been hampering the transition to clinical trials. Only recently, a more efficient production system of soluble endostatin in *Pichia pastoris* was reported (6). However, the challenge of the long-term storage of bioactive protein and the cumbersome daily administration remain. In this paper, we demonstrated that a single injection of ADV.mEnd resulted in persistent high endostatin serum levels (936 ng/ml), more than 30 times higher

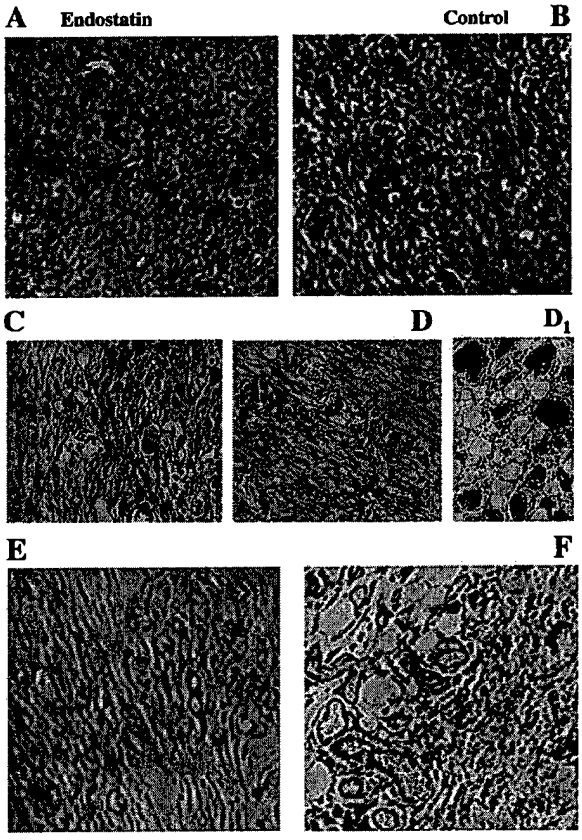

Fig. 6. Reduced angiogenesis in ADV.mEnd-treated tumors. (*A–F*) Decreased tumor vascularization could be demonstrated in all ADV.mEnd-treated tumors [LLC, hematoxylin/eosin (*A*); JC, hematoxylin/eosin (*C*); JC, ICAM-1 (*E*)] as compared with the controls [LLC, hematoxylin/eosin (*B*); JC, hematoxylin/eosin (*D*); JC, ICAM-1 (*F*)]. (D_1) Markedly increased angiogenesis also could be observed at the border zone between tumor and surrounding tissue in the control group of the JC tumors. (*A–F*, ×100; D_1, ×200.)

than previously was reported with nonviral endostatin gene therapy (7, 8). The activity (ED_{50}) of ADV.mEnd-expressed and purified endostatin in endothelial cell proliferation assays was similar (15) or slightly lower (5) than that of endostatin obtained through other expression systems. Higher endostatin activity of recombinant endostatin was reported in only one paper, where a different *in vitro* assay (endothelial cell migration) and *in vivo* administration (peritumoral) were used (16). The adenovirus-mediated delivery of endostatin was clearly superior (much higher transgene expression levels, treatment of larger preestablished tumors, and complete prevention of metastases) to the reported plasmid-based, nonviral gene transfer methods (direct i.m. injection or liposome complexed) (7, 8). A positive correlation between endostatin dose and inhibition of endothelial cell proliferation *in vitro* has been demonstrated (5, 17). Furthermore, it also has been reported that levels of <1 μg/ml endostatin did not induce endothelial cell apoptosis *in vitro* (17). Thus, the better therapeutic effect of the endostatin protein treatment as compared with the ADV.mEnd therapy may be caused by still insufficient expression levels of endostatin (≈ 1 μg/ml) by the adenovirus. Serum levels of endostatin after protein therapy have not been reported thus far. However, the effective dose of recombinant endostatin protein for tumor inhibition (10–20 mg/kg per day) results probably in higher circulating endostatin levels than reported here, even when the relatively short half-life of the protein is taken into account.

As recently shown in a transgenic multistage model of carcinogenesis, endostatin was not able to prevent the transition from large adenomas into the invasive carcinoma stage (18). This occurrence could be the reason that, despite the early statistically significant treatment effect of ADV.mEnd (6 days after virus injection), insufficient endostatin levels may have allowed a number of cells to "escape" from therapy and to enter an invasive state where endostatin was not effective anymore. As previously demonstrated, lung metastases could be inhibited very effectively with a 50 times lower dose of recombinant endostatin (0.3 mg/kg) than that used for tumor treatment (5). Therein may be the explanation as to why the ADV.mEnd-generated endostatin levels, albeit not high enough to induce regression of preestablished tumors, were sufficient to completely prevent the engraftment of circulating tumor cells to form lung micrometastases. In contrast, only a partial effect on metastases was observed when a low-expression gene delivery system was used (7). This finding provides important information, especially for potential clinical applications, on the impact of endostatin serum concentrations on primary tumors and metastases.

Circulating endostatin levels, however, may not be the only explanation for the difference between therapy with endostatin protein and with ADV.mEnd, because angiogenesis is a complex process involving proteolytic basement membrane degradation as well as loss of adhesion, migration, and proliferation of endothelial cells. Different tissue deposition patterns between the s.c. administered protein, and the mostly hepatically produced and secreted endostatin by adenovirus gene therapy may play a role in the control of tumor angiogenesis, because it has been suggested that tissue saturation at the tumor site after repetitive treatments could also be an important factor (2, 19). Despite the known facts about endostatin [crystal structure (20), induction of endothelial cell apoptosis (21), interaction with other extracellular matrix proteins (22) and heparan sulfate (23), and some mutational analyses (15, 16, 24)], much of the molecular mechanism of its action, including the role of zinc (16, 24), remains elusive.

In summary, we showed that high circulating levels of biologically active endostatin could be achieved through adenovirus-mediated gene transfer. To increase the efficacy of endostatin gene therapy, however, an increase of transgene expression levels by choosing different promoters/enhancers and/or by generation of fusion proteins to prolong the endostatin half-life are needed. Further studies on the molecular mechanism of endostatin may also provide new insights that could be explored for endostatin gene therapy. Finally, a gutless, nonimmunogenic, and less toxic adenovirus system that was shown to permit stable long-term transgene expression in monkeys would have to be explored for potential human trials (25). In the present form, endostatin gene therapy does not provide a cure for cancer; however, it may be very useful in conjunction with other cancer treatment modalities.

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Adenovirus-Mediated Gene Transfer of Endostatin Reduces Tumor Vasculature and Changes Tumor Vessel Composition

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Inhibition of angiogenesis by endostatin gene transfer has been shown to be an effective strategy in cancer therapy in mice. However, in addition to the reduction of the quantity of tumor vasculature qualitative effects on composition and degree of tumor vessel maturation might also play an important role in the therapeutic success. To test this hypothesis, we systematically evaluated the composition of tumor vasculature in a nude mice breast cancer model comparing mice treated by endostatin gene transfer (group A, n=5) to control vector treated animals (group B, n=5). We have constructed a recombinant adenovirus expressing murine endostatin that is biologically active both *in vitro* and *in vivo*. Persistent high serum levels of endostatin (605-1740 ng/ml; mean 936 ng/ml) were achieved after systemic administration of the vector to nude mice which resulted in significant reduction of the growth rates and the volumes of JC breast carcinoma ($p<0.001$). Tumor tissue was formalin fixed and processed for HE and immunohistochemical staining detecting α-actin and von Willebrand factor (vWF) protein. Quantitative morphometric analysis was performed. Overall mean vessel area per field was significantly lower in group A ($p=0.004$). In addition, the endostatin vector treatment nearly completely prevented the formation of α-actin and vWF positive tumor vessels ($p=0.0001$) As a result tumor vessel composition was significantly changed. Our data indicate effects of endostatin protein not only on initiation of tumor vessel growth but also on maturation and differentiation.

Endostatin gene transfer inhibits tumor vessel maturation proportionally to transgene expression levels

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Background: Inhibition of angiogenesis by endostatin gene transfer has been shown to be an effective strategy in cancer therapy in mice. Endostatin is known to reduce the number of tumor vessels, however, potentially important effects of endostatin therapy on tumor vessel maturation have not been investigated. *Methods and Results:* To investigate the effect of endostatin on tumor angiogenesis, we first constructed a recombinant adenovirus expressing murine endostatin that was tested for biological activity both *in vitro* and *in vivo*. We then systematically evaluated the composition of tumor vasculature in a nude mice breast cancer model comparing mice treated by endostatin gene transfer (group A, n=10) with control vector treated animals (group B, n=10). Persistent high serum levels of endostatin (605-1740 ng/ml; mean 936 ng/ml) were achieved after systemic administration of the vector to nude mice which resulted in significant reduction of the growth rates and the volumes of JC breast carcinoma. Tumor tissue was formalin fixed and processed for HE and immunohistochemical staining detecting alpha-actin and CD 31 protein. Quantitative morphometric analysis was performed. Overall mean vessel area per field was significantly lower in the endostatin treated group ($p<0.05$). In addition, the endostatin vector treatment almost completely prevented the formation of alpha-actin positive tumor vessels compared to controls ($p=0.01$). Most interestingly, only the density of alpha-actin positive tumor vessels, but not the total number of tumor vessels, correlated negatively with endostatin transgene levels of individual animals ($r=-0.58$, $p=0.018$). As a result tumor vessel composition as indicated by the ratio of alpha-actin positive to overall tumor vessel density was significantly changed. *Conclusions*: Our data indicate that endostatin affects not only the number of tumor vessels but also induces a dose dependent shift from alpha-actin positive to alpha-actin negative tumor vessels. This strongly suggests that endostatin influences the complex process of vessel maturation possibly by interfering with vessel pruning or pericyte recruitment.

Transcriptional Targeting of Conditionally Replicating Adenovirus to Dividing Endothelial Cells

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SUMMARY

Conditionally replicating adenoviruses (CRADs) are a novel strategy in cancer treatment and clinical trials using CRADs targeted to tumor cells have been reported recently. We hypothesized that it would be possible to construct CRADs targeted to dividing endothelial cells, which are present in the tumor endothelium. We utilized the regulatory elements of Flk-1 and endoglin genes, which have been shown to be highly overexpressed in angiogenic endothelial cells, to construct two CRADs: Ad.Flk-1, which has adenoviral E1A gene under the control of the Flk-1 enhancer/promoter, and Ad.Flk-Endo, which harbors the same Flk-1 enhancer/promoter as Ad.Flk-1, plus it has the adenoviral E1B gene under control of the endoglin promoter. Viral titer measurements by plaque assay showed that in human umbilical vein endothelial cells (HUVECs), both CRADs replicated at levels comparable to that of wild-type adenovirus. In Flk-1 and endoglin negative Hep3B and A549 cells, however, the replication of Ad.Flk-1 and Ad.Flk-Endo was reduced by 30-fold and 600-fold, respectively. Cytotoxicity assays demonstrated that both CRADs killed HUVECs as effectively as wild-type adenovirus and their cytotoxicity in Hep3B and A549 cells was comparable to nonreplicating control adenovirus. Furthermore, there was a striking inhibition (83% - 91%) of capillary network formation in an *in vitro* angiogenesis assay when HUVECs were infected with Ad.Flk-1 or Ad.Flk-Endo as compared to the nonreplicating control virus. These results demonstrate that CRADs can be transcriptionally targeted to dividing endothelial cells with high specificity, and that the combined use of Flk-1 and endoglin regulatory elements has a synergistic effect on targeting specificity. This principle may be

incorporated into novel therapeutic agents to develop anti-angiogenic treatment for cancer.

Keywords: adenovirus, gene therapy, transcriptional targeting, anti-angiogenesis, cancer treatment

INTRODUCTION

Conditionally replicating viruses are a novel approach to cancer treatment and the focus of intense study.[1,2] Most initial cancer gene therapy experiments have been carried out using replication-defective viruses, which are by definition limited in their ability to infect and spread to neighboring cancer cells. Replication-selective oncolytic viruses provide an attractive alternative to cancer treatment, as they are able to replicate in tumor cells and spread to neighboring cells upon cell lysis. In addition to conditionally replicating adenoviruses (CRADs), herpes simplex virus-1, vaccinia virus, reovirus and Newcastle Disease virus have been tested for oncolytic purposes in clinical trials.[3] Two main strategies have been utilized to construct tumor-targeted CRADs. The first one involves the use of tumor- or tissue-specific promoters, such as MUC1, AFP, PSA, kallikrein-2 and pS2 to drive adenoviral genes that are essential for replication.[4-8] This approach has been successful in animal experiments and the prostate-specific viruses CN-706 and CV-787 are being tested in clinical trials. The second strategy is based on deleting adenoviral genes that become dispensable in cancer cells, such as the E1A and E1B genes responsible for inactivating tumor suppressor p53 and Rb genes, thereby enabling the virus to selectively replicate in tumor cells.[9,10] This strategy has also been effective *in vivo* and some level of efficacy has also been reported in a clinical trial combining the E1B 55kDa-deleted Onyx-015 virus with chemotherapy.[11] However, a major limitation of tumor-targeted CRADs is their relatively poor efficiency to spread in the tumor mass, thereby requiring repeated viral injections administered at multiple sites and over several days.[11,12]

While most endothelial cells are normally quiescent, angiogenesis, the formation of new blood vessels is essential for certain physiological processes such as female reproductive cycle and wound healing. In addition, many studies have shown that tumor growth is dependent on angiogenesis, thus providing a rationale for anti-angiogenic therapy for cancer.[13,14] Angiogenesis is a complex multicomponent process, involving many growth factors and their receptors, cytokines, proteases and adhesion molecules, providing multiple targets for therapeutic intervention.[15,16] In addition, many endogenous proteins or protein fragments have been shown to have anti-angiogenic properties.[13,17,18] Targeting a CRAD to the tumor endothelium offers a novel approach to anti-angiogenic therapy for cancer.

Targeting the vessels of the tumor instead of the tumor itself potentially offers enhanced viral spread via the tumor's blood supply. In addition, as the target is the genetically normal endothelial cell, resistance to treatment due to somatic mutations in the target cell does not occur. In order to transcriptionally target the dividing endothelial cell, promoters of genes with specificity for these cells can be utilized. Flk-1 (also called KDR and VEGFR-2) is a high-affinity tyrosine kinase receptor for the angiogenic growth factor, VEGF. Flk-1 is essential for endothelial cell differentiation and vasculogenesis and is expressed by endothelial cells or their precursors during development.[19,20] Flk-1 expression is suppressed in adult endothelium, but is highly induced in the newly formed blood vessels in a variety of human tumors.[19,21,22] In a recent Flk-1 promoter characterization study a 939-bp promoter fragment in combination with a 510-bp enhancer element in the first intron specifically targeted lacZ expression to angioblasts

and endothelial cells of transgenic mice.[23] When various tumors were grown in these mice, strong tumor endothelium-specific reporter gene expression was observed, while being absent from most blood vessels in normal adult tissue.[24] Endoglin (CD105) is a cell surface component of the TGF-β receptor complex, which is preferentially expressed by endothelial cells. A detailed expression study demonstrated that endoglin expression was strongly elevated in the angiogenic endothelium and that endoglin expression correlated with cell proliferation markers.[25] A 741-bp fragment of the endoglin promoter displayed tissue-specific activity in dividing human and bovine endothelial cells.[26]

The goal of the current study was to determine whether replicating adenoviruses could be targeted to dividing endothelial cells. To this end, we constructed two CRADs targeting the dividing endothelial cells via Flk-1 and endoglin regulatory elements.

RESULTS

Construction of Recombinant Ad.Flk-1 and Ad.Flk-Endo Vectors

In order to construct an adenovirus that replicates selectively in Flk-1 positive cells, plasmid pFlk-1 was created by deleting the proximal adenovirus E1A promoter (bp 357 to 547) from pXC1 containing adenovirus 5 sequences from bp 22 to 5790 and replacing it with the 1433 bp Flk-1 enhancer/promoter construct. To construct a virus that replicates selectively in Flk-1 and endoglin positive cells, plasmid pFlk-Endo was constructed by

inserting the 741bp Endoglin promoter at bp 1691 of pFlk-1, before the transcription start site of the adenovirus E1B gene. Adenoviruses Ad.Flk-1 and Ad.Flk-Endo were produced by co-transfecting the E1-complementing 293 cells with backbone plasmid pBHG10, which harbors the rest of the adenoviral genome except for a deletion of the E3 gene region, with plasmids pFlk-1 and pFlk-Endo, respectively (Fig 1). The genome lengths of Ad.Flk-1 and Ad.Flk-Endo are 97% and 99% of wild-type Ad5, respectively.

Selection and Characterization of Target Cells

Human primary cells and cell lines were used to assess the selectivity of Ad.Flk-1 and Ad.Flk-Endo viral replication and cytotoxicity. HUVECs represent the dividing endothelial cell population and as expected, RT-PCR analysis demonstrated high levels of both Flk-1 and endoglin expression (Fig 2). Therefore, these cells could be used as target cells in evaluating the efficiency of Ad.Flk-1 and Ad.Flk-Endo. In contrast, no Flk-1 or endoglin expression was detected in Hep3B and A549 cells, making these cell lines suitable negative controls (Fig 2). As very little Flk-1 or endoglin is expressed *in vivo* outside the tumor endothelial cell population, Hep3B and A549 cells can be used to analyze the cytotoxicity of the viruses in non-target cells.[19,22,27]

In order to analyze the adenoviral transduction efficiency of the different cell types used, HUVECs, Hep3B and A549 cells were transduced with increasing concentrations of E1-deleted β-gal virus and the transduction efficiency was determined by β-gal staining (data not shown). Hep3B cells were transduced most efficiently, with viral doses of $2x10^5$ pfu/ml and $2x10^6$ pfu/ml resulting in transduction efficiencies of 10% and 30%,

respectively. A ten-fold increase in viral dose was required to achieve comparable transduction efficiencies in A549 cells, with viral doses of $2x10^6$ pfu/ml and $2x10^7$ pfu/ml resulting in transduction efficiencies of 10% and 20%, respectively. HUVECs were the most difficult to transduce, with viral doses of $2x10^7$ pfu/ml and $2x10^8$ pfu/ml resulting in transduction efficiencies of 10% and 25%, respectively. The viral concentrations in subsequent experiments were adjusted accordingly, allowing direct comparison for the effect of transcriptional targeting.

Selective Replication of Ad.Flk-1 and Ad.Flk-Endo

From the oncolysis point of view, the most important quality of conditionally replicating viruses is their ability to productively and selectively replicate in the target cells. To assess the selective viral replication, HUVECs, A549 and Hep3B cells were infected by Ad.Flk-1, Ad.Flk-Endo and wild-type Ad5, and viral progeny production was measured by a series of plaque assays. In HUVECs, Ad.Flk-1 and Ad.Flk-Endo viruses replicated at levels comparable to that of wild-type Ad5. In Hep3B and A549 cells, however, the viral titer of Ad.Flk-1 was reduced approximately 30-fold. Interestingly, Ad.Flk-Endo had an even higher specificity ratio, with replication levels reduced 120-fold in A549 and 600-fold in Hep3B cells (Fig. 3). The high replication specificity of Ad.Flk-Endo demonstrates that the combined use of Flk-1 and endoglin regulatory elements has a synergistic effect on targeting specificity.

Targeted Cell Lysis by Ad.Flk-1 and Ad.Flk-Endo

To evaluate if selective replication of Ad.Flk-1 and Ad.Flk-Endo would translate into selective cytotoxicity, a series of trypan blue exclusion assays was performed. HUVECs, Hep3B and A549 cells were infected with Ad.Flk-1 and Ad.Flk-Endo, while wild-type and E1-deleted β-gal viruses were used as positive and negative controls, respectively. At 48 hours postinfection, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus showed marked reduction of living cells as compared with cells transduced with E1-deleted β-gal virus (Fig. 4). At 72 hours the difference was even clearer, with almost complete cytotoxicity in cells infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus. Ad.Flk-Endo was somewhat more cytotoxic than Ad.Flk-1 at both time points. In contrast, a clear difference between the cytotoxicity of wild-type and conditionally replicating viruses was observed in Flk-1 and endoglin negative Hep3B and A549 cells. The wild-type virus efficiently killed most Hep3B and A549 cells already at 48 hours postinfection, whereas Ad.Flk-1 and Ad.Flk-Endo caused only minimal cytotoxicity. At 72 hours, when viral replication had progressed, the difference was more profound, in Hep3B cells infected with Ad.Flk-1 and Ad.Flk-Endo the number of living cells was 17.3 and 18.8 times higher, respectively, when compared to cells infected with wild-type virus. Similar ratios were observed for A549 cells, with the number of living cells infected with Ad.Flk-1 and Ad.Flk-Endo being 22.6 and 22.0 times higher, respectively, when compared to cells infected with wild-type virus at 72 hours (Fig. 4). Although some cytotoxicity was observed when comparing Ad.Flk-1 and Ad.Flk-Endo to E1-deleted β-gal virus in Hep3B and A549 cells, the difference was not statistically significant. The cytotoxicity of CRADs on a particular cell type depends not only on the rate of

replication but also on the infectivity of the cell.[28] This was demonstrated when Hep3B cells, which are very efficiently infected by Ad5, were infected with $2x10^7$ pfu/ml of Ad.Flk-1 and Ad.Flk-Endo, which resulted in a 60% transduction efficiency, cytotoxicity was observed.

Cytopathic effect was also visualized by examining infected Hep3B and HUVEC monolayers (Fig. 5). Infection of HUVECs with Ad.Flk-1, Ad.Flk-Endo and wild-type virus caused profound cytotoxicity at 48 hours postinfection with rounding and detachment of almost all cells (Fig. 5). In contrast, infection of Hep3B control cells with Ad.Flk-1 and Ad.Flk-Endo caused no discernible cytotoxicity at 48 hours postinfection, whereas infection of Hep3B cells with the same concentration of wild-type virus caused complete cytotoxicity. The results demonstrate that the selective replication of Ad.Flk-1 and Ad.Flk-Endo translated also into specific cell killing of the target cells.

Effect of Ad.Flk-1 and Ad.Flk-Endo on Capillary Tube Formation

Angiogenesis is a complex process characterized by endothelial cell proliferation, differentiation and migration. In order to evaluate the effect of Ad.Flk-1 and Ad.Flk-Endo infection on these processes, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo and control viruses were plated on wells precoated with Matrigel basement membrane matrix. 8 hours after plating, HUVECs infected with the E1-deleted β-gal virus as well as uninfected cells were able to form a network of capillary-like structures and by 24 hours after plating these structures covered most of the well. In contrast, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo or wild-type virus failed to differentiate (Fig. 6). When

compared with cells infected with E1-deleted β-gal virus the number of capillary-like structures was 91% and 83% less with cells infected with Ad.Flk-1 and Ad.Flk-Endo, respectively. These results were statistically significant ($p<0.01$) and there was no statistical significance in the inhibition efficiency between Ad.Flk-1, Ad.Flk-Endo and wild-type virus. Thus, infection with Ad.Flk-1 or Ad.Flk-Endo efficiently inhibits angiogenesis *in vitro*.

DISCUSSION

In this communication we describe two novel adenoviruses, Ad.Flk-1 and Ad.Flk-Endo, which target the expression of adenoviral E1A and E1B genes to dividing endothelial cells by Flk-1 and endoglin promoters, respectively. We show that Ad.Flk-1 and Ad.Flk-Endo possess significant differential replication ratios in Flk-1 and endoglin positive cells as compared with cells where these genes are not expressed. Flk-1 is an endothelial-cell specific gene, which in contrast to other endothelial genes that are expressed constitutively, is absent from most vascular beds of the adult organism but is strongly and specifically induced in the dividing endothelial cells of the tumor endothelium.[19,22] Endoglin has a similar expression pattern with selective expression in endothelial cells and strong upregulation especially in the endothelium of the tumor edges, where active cell division occurs.[25,29] Some endoglin expression has also been reported in cells of the hematopoietic lineage.[29] Fortunately, hematopoietic stem cells and erythroid precursors

are highly resistant to adenovirus infection.[30,31] Therefore, a therapeutic agent targeting Flk-1 and endoglin positive cells should be highly specific for tumor vasculature.

We chose E1A as the target for transcriptional regulation because it has been shown to be the major regulator of adenoviral replication. E1B was chosen to further increase specificity as it is also essential for proper viral replication and, importantly, the expression levels of E1B do not depend on E1A, thereby allowing adenoviral replication to be regulated by two independent elements.[32] Similar strategies have previously been utilized in constructing viruses which target prostate, breast and liver tumor cells.[4-8] However, no replicating viruses targeting the dividing endothelial cells have previously been reported.

In HUVECs, the replication of Ad.Flk-1 and Ad.Flk-Endo was increased 30-fold and 600-fold, respectively, when compared to Flk-1 and endoglin negative control cells (Fig. 3). These selective replication ratios are comparable to those obtained with previously reported tumor-targeted CRADs. The high specificity of Ad.Flk-Endo demonstrates that the use of two separate regulatory elements has a synergistic effect on targeting specificity. A similar increase in specificity has been reported for CV764, which has two separate prostate carcinoma-specific promoters driving E1A and E1B.[7] Ad.Flk-1 and Ad.Flk-Endo were also able to cause selective cytotoxicity as they killed Flk-1 and endoglin positive HUVECs as efficiently as wild-type virus, but in Flk-1 and endoglin negative Hep3B and A549 cells the wild-type virus was approximately 20 times more cytotoxic. It is notable that the therapeutic ratios of many conventional cytotoxic drugs

range from 1.5:1 to 6:1.[33] Furthermore, there was no statistical significance when the cytotoxicity of Ad.Flk-1 and Ad.Flk-Endo was compared to the E1-deleted control virus (Fig. 4). These results clearly demonstrate that Ad.Flk-1 and Ad.Flk-Endo are able to specifically replicate in, and kill, Flk-1 and endoglin positive cells. It is still possible, however, that some cytotoxicity can be caused by leakiness of the Ad.Flk-1 and Ad.Flk-Endo promoter constructs, especially in cell lines that are very efficiently infected by Ad5.

As angiogenesis is a complex process characterized by endothelial cell proliferation, differentiation, invasion and migration, the effect of Ad.Flk-1 and Ad.Flk-Endo on these processes was also evaluated in Matrigel assays, which mimic angiogenesis *in vitro*. Ad.Flk-1 and Ad.Flk-Endo were very effective in hindering the angiogenic process and it is notable that they inhibited angiogenesis already at 32 hours postinfection, well before any cytotoxicity due to viral replication could be observed. This indicates that these viruses efficiently inhibit angiogenesis, at least *in vitro*, already at low levels of viral replication.

The development of replication-competent vectors is an attractive alternative strategy to using replication-defective viruses in cancer treatment. By specifically replicating in the target cells, CRADs deliver the therapeutic vector at the intended site of action, keeping the therapeutic dose to a minimum.[34] However, the efficacy of CRADs targeting tumor cells has thus far been limited, due at least in part to limited spread of the virus in solid tumor tissues. CRADs targeting the tumor endothelium instead of the tumor itself should

be able to spread in the tumor via its blood supply. Furthermore, as the target cell is genetically stable, therapy resistance due to somatic mutations is not anticipated. Other advantages of endothelial targeting include easy reach of target tissue upon intravenous administration and the amplification effect on tumor killing, as one endothelial cell is known to support the nutritional needs of approximately 100 tumor cells.[35] The amplification effect was elegantly illustrated in a recent report comparing the anti-tumor efficacies of tumor vs. tumor endothelium infecting retroviruses expressing the herpes simplex virus-thymidine kinase (HSV-tk) cytotoxic gene. The therapeutic effect in tumors with HSV-tk infected vasculature comprising less than 5% of the total tumor mass was similar to that of tumors with HSV-tk infected tumor cells comprising over 46% of the total tumor mass.[36] This is an encouraging result for targeted anti-angiogenic strategies such as described here as it indicates that infection of relatively few tumor endothelial cells results in a strong anti-tumor effect.

The oncolytic effect of CRADs on a particular cell type depends not only on the rate of replication but also on the infectivity of the cell.[28] As tumor endothelial cells are not very susceptible to Ad5 infection, the CRADs described here are best suited for local or regional (e.g. selective canulation of tumor-feeding vessels) administration *in vivo*. In order to improve the selectivity and efficacy of transcriptionally targeted viruses like Ad.Flk-1 and Ad.Flk-Endo, they can be further optimized by combining them with transductional targeting e.g. through genetic modification of the viral capsid. Recent advances in adenovirus and tumor biology should make such targeting possible. The binding site of the Coxsackie- and Adenovirus Receptor (CAR) on the adenovirus fiber,

which is a major determinant of adenovirus tropism, has recently been described.[37] It was also demonstrated that by deleting this binding site and adding a targeting ligand to the adenovirus fiber, the modified vector could be successfully retargeted.[37] In order to retarget Ad.Flk-1 and Ad.Flk-Endo, ligands homing to sites of neoangiogenesis must be used. Such ligands have been recently discovered, thereby making it possible to construct CRADs targeting the tumor endothelium by both transcriptional and transductional mechanisms.[38,39] The construction of such viruses is under way in our laboratory.

In summary, we demonstrate here for the first time the feasibility of targeting replication-competent adenoviruses to dividing endothelial cells with great specificity. This principle has the potential to improve gene therapy strategies directed at disseminated tumors.

MATERIALS AND METHODS

Construction of Ad.Flk-1 and Ad.Flk-Endo

Plasmid pXC1, which contains human adenovirus 5 sequences from bp 22 to 5790, was purchased from Microbix (Microbix Biosystems Inc., Toronto, Canada). For insertion of the Flk-1 enhancer/promoter a unique AgeI site was created in the adenovirus E1A promoter of pXC1 by overlapping PCR as follows. The first primer pair (TCGTCTTCAAGAATTCTCATG (sense) and TTTCAGTCACCGGTGTCGGA (antisense)) produced a PCR fragment from the unique EcoRI site in the PBR322

backbone of pXC1 to the new AgeI site at position 547. The second primer pair (TCCGACACCGGTGACTGAAA (sense) and GCATTCTCTAGACACAGGTG (antisense)) produced a PCR fragment from the new AgeI site to a unique XbaI site at position 1339. Combining equal amounts of the two PCR products, a third PCR was performed with the two outside primers, cut with EcoRI and XbaI, and cloned into similarly cleaved pUC19 to yield pUCE1A. This plasmid was cut with unique enzymes SacII at position 357 and AgeI at position 547 to delete the endogenous adenovirus E1A promoter.

In order to amplify the Flk-1 enhancer and promoter liver tissue from a BALB/c mouse was homogenized, and DNA was extracted using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). A 923-bp Flk-1 promoter element was amplified using specific primers based on published sequence: ATTTAGCGGCCGCAGTTCACAACCGAAATGTCTTC (sense primer with NotI linker) and AGTTTACCGGTATCCTGCACCTCGCGCTG (antisense primer with AgeI linker).[40] A 510-bp Flk-1 enhancer element was amplified using specific primers based on published sequence: TCCCCGCGGTAAATGTGCTGT-CTTTAGAAG (sense primer with SacII linker) and AATATGCGGCCGCTCCAA-TAGGAAAGCCCTTC (antisense primer with NotI linker).[23] The promoter and enhancer fragments were cut with NotI-AgeI and SacII-NotI, respectively, and cloned into similarly cut pUCE1A to yield pUCE1A-Flk1. The modified E1A fragment containing the Flk-1 enhancer/promoter was released from pUCE1A-Flk1 by EcoRI-XbaI digestion and cloned into EcoRI-XbaI digested pXC1 to yield pFlk-1 (Fig. 1).

In order to construct pFlk-Endo a unique BlpI restriction was created in pFlk-1 by overlapping PCR as follows. The first primer pair (TCACCTGTGTCTAGAGAATGC (sense) and GTAACCAAGCTTAGCCCACG (antisense)) produced a PCR fragment from the unique XbaI site of pFlk-1 to the new BlpI site at position 1690 of adenovirus sequence. The second primer pair (CGTGGGCTAAGCTTGGTTAC (sense) and CCAGAAAATCCAGCAGGTACC (antisense)) produced a PCR fragment from the new BlpI site to a unique KpnI site of pFlk-1. Combining equal amounts of the two PCR products, a third PCR was performed with the two outside primers, cut with XbaI and KpnI, and cloned into XbaI-KpnI digested pFlk-1 to yield pFlk-BlpI, which now has a unique BlpI site before the transcription start site of E1B.

In order to amplify the endoglin promoter human DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen). A 741-bp endoglin promoter was amplified using specific primers based on published sequence: GATCATGCTAAGCGATCCCAGCGCTACCATCTTC (sense primer with BlpI linker) and TATAATGCTTAGCGTGGGGGCCTGTGCGCTGG (antisense primer with BlpI linker).[26] The promoter fragment was cut with BlpI and cloned into similarly cleaved pFlk-1-BlpI to yield pFlk-Endo (Fig. 1). The sequence of all PCR fragments in pFlk-1 and pFlk-Endo were verified by sequencing.

Recombinant adenoviruses Ad.Flk-1 and Ad.Flk-Endo were prepared by co-transfecting 293 cells with plasmids pFlk-1 and pFlk-Endo with backbone plasmid pBHG10 (Microbix), which contains an E1 deletion of bp 188-1339 and an E3 deletion of bp

28133-30818, as described.[41] Recombinant adenovirus was isolated from a single plaque, expanded in 293 cells and purified by double cesium gradient ultracentrifugation. The viral particles were measured by optical absorbance at 260 nm, and the plaque-forming units (pfu) were determined by standard agarose-overlay plaque assay on 293 cells. The genome lengths of Ad.Flk-1 and Ad.Flk-Endo are 97% and 99% of wild-type Ad5, respectively.

Cells and Viruses

Hep3B, A549 and 293 cell lines were obtained from American Type Culture Collection. The cells were cultured in DMEM medium supplemented with 10% FBS, 0.2 mM glutamine, and penicillin/streptomycin. In addition, Hep3B medium was supplemented with 1 mM sodium pyruvate. HUVECs were isolated from umbilical cords (Institutional Review Board-approved cord blood program) by collagenase type IV (Sigma-Aldrich Inc., St Louis, MO) perfusion (0.2% in Hanks' balanced salt solution) for 20 minutes at room temperature. The cells then were cultured in EGM-2 medium (Clonetics Corporation, Walkersville, MD). The E1-deleted β-gal virus was produced in our vector core laboratory by co-transfecting pCMV-lacZ shuttle plasmid with pBHG10 backbone plasmid as described above.

RT-PCR

RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen). RT-PCR was performed with OneStep RT-PCR kit (Qiagen) using 0.6 μg template total RNA and

specific primers for Flk-1 and endoglin cDNA. Specific primers for G3PDH housekeeping gene were included in each reaction as a positive control. The samples were run on a 1% agarose gel and photographed.

In Vitro Replication Assay

$2x10^5$ cells/well were plated on 6-well dishes 24 hours before infection. Cells were infected with 2x107 pfu/ml (HUVEC), 2x106 pfu/ml (A549) or 2x105 pfu/ml (Hep3B) of virus in 1 ml serum-free medium. After a 2 hour incubation at 37°C, cells were washed once with prewarmed PBS and 2 ml complete medium was added to each well. After an additional 48 hours, the cells were scraped into the culture medium, and lysed by three freeze-thaw cycles. The lysate was centrifuged at 3000 rpm for 5 minutes and the supernatant was analyzed for virus production by duplicate plaque assay on 293 cells.

Trypan Blue Exclusion Assay

$5x10^4$ cells/well were plated on 24-well plates 24 hours before infection and infected as described above. 48 hours or 72 hours later the medium was removed and attached cells were detached by trypsin. The cells were stained with trypan blue and live cells were counted using a hemacytometer.

In Vitro Angiogenesis Assay

Matrigel basement matrix (Becton Dickinson, Bedford, MA) was diluted 1:2 in cold DMEM medium. 200 µl diluted Matrigel was plated into 24-well plates and allowed to

gel for 30 minutes at 37°C before cell seeding. HUVECs were plated on 6-well plates at a density of $2x10^5$ cells/well 24 hours before infection and infected at a concentration of $2x10^8$ pfu/ml as described above. 24 hours after infection the cells were collected with trypsin/EDTA, resuspended in EGM-2 medium and plated at a density of $5x10^4$ cells/well on 24-well plates precoated with Matrigel as described above. After 24 hours incubation the plates were photographed and quantitated. Capillary tubes were defined as cellular extensions linking cell masses or branch points.

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FIGURE LEGENDS

Fig 1. A schematic representation of Ad.Flk-1 and Ad.Flk-Endo. Numbers above the drawings indicate positions in adenovirus 5 sequence. Numbers inside the boxes indicate sizes of the regulatory elements. Both viruses harbor a deletion of the endogenous E1A promoter and the E3 region, indicated by hatched boxes. Flk-1 E = Flk-1 enhancer, Flk-1 P = Flk-1 promoter, Endoglin P = endoglin promoter.

Fig 2. RT-PCR analysis of Flk-1 and Endoglin expression on HUVECs, A549s and Hep3Bs. RT-PCRs were performed using 0.6 μg template RNA from cultured cells and specific primers for Flk-1 and endoglin cDNA. Specific primers for G3PDH housekeeping gene were included in each reaction as a positive control. Arrows indicate the 500 bp, 410 bp and 109 bp amplified products of Flk-1, endoglin and G3PDH, respectively.

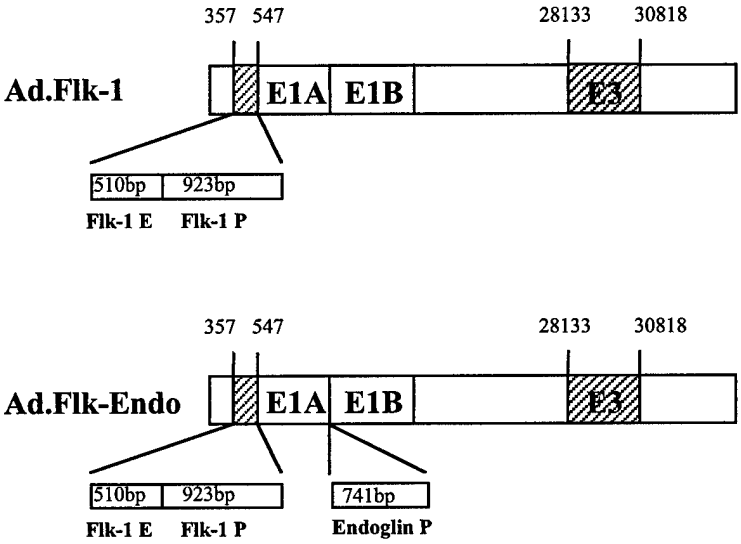
Fig 3. Viral replication of Ad.Flk-1, Ad.Flk-Endo and wild-type virus in Flk-1/endoglin positive (HUVEC) and negative (A549 and Hep3B) human cells. Monolayers in 6-well plates were infected at a concentration of $2x10^7$ pfu/ml (HUVEC), $2x10^6$ pfu/ml (A549) or $2x10^5$ pfu/ml (Hep3B). Viral titers were assessed by plaque assay on 293 cells.

Fig 4. Cytotoxicity of Ad.Flk-1 and Ad.Flk-Endo. Monolayers in 24-well plates were infected at a concentration of $2x10^7$ pfu/ml (HUVEC and A549) or $2x10^5$ pfu/ml (Hep3B). Cells infected with wild-type and E1-deleted β-gal viruses and uninfected cells were used controls. 48 hours or 72 hours later cells were stained with trypan blue and live cells were counted. * $p<0.05$, ** $p<0.01$

Fig 5. Cytopathic effects associated with Ad.Flk-1 and Ad.Flk-Endo infection. Hep3B and HUVEC monolayers in 24-well plates were infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus at a concentration of $2x10^8$ pfu/ml (HUVEC) or $2x10^5$ pfu/ml (Hep3B). 48 hours later the cells were photographed.

Fig 6. The effect of Ad.Flk-1 and Ad.Flk-Endo infection on HUVEC differentiation into capillary-like structures. HUVEC monolayers on 6-well plates were infected with Ad.Flk-1, Ad.Flk-Endo, wild-type and E1-deleted β-gal virus at a concentration of $2x10^8$ pfu/ml. 24 hours later the infected cells and uninfected controls were seeded on Matrigel and the spontaneous formation of capillary tubes was recorded at 24 hours after plating and quantitated as described. * $p<0.05$, ** $p<0.01$

Figure 1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547
28133
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E1B
E3
510bp
923bp
Flk-1 E
Flk-1 P
357
547
28133
30818
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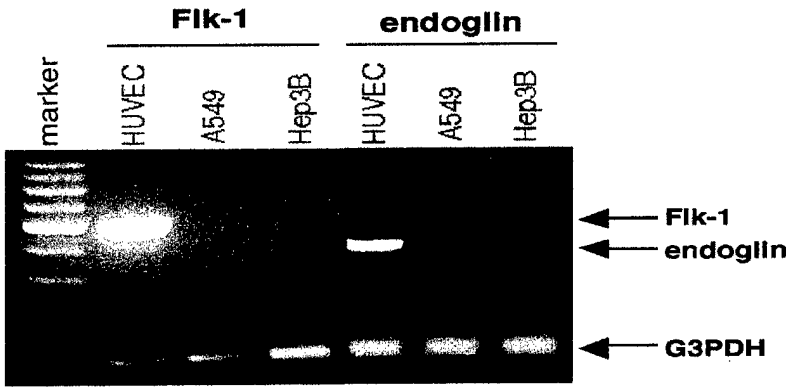
Endoglin P

Figure 2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A549
Hep3B
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Hep3B
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endoglin
G3PDH

Figure 3

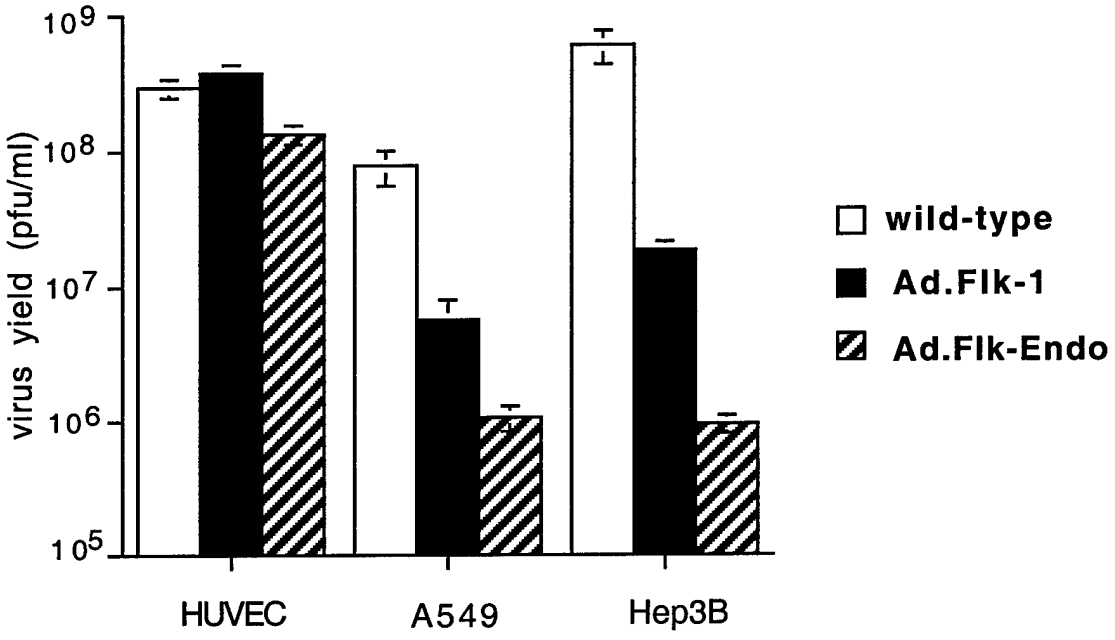

virus yield (pfu/ml)
10^9
10^8
10^7
10^6
10^5
HUVEC
A549
Hep3B
wild-type
Ad.Flk-1
Ad.Flk-Endo

Figure 4

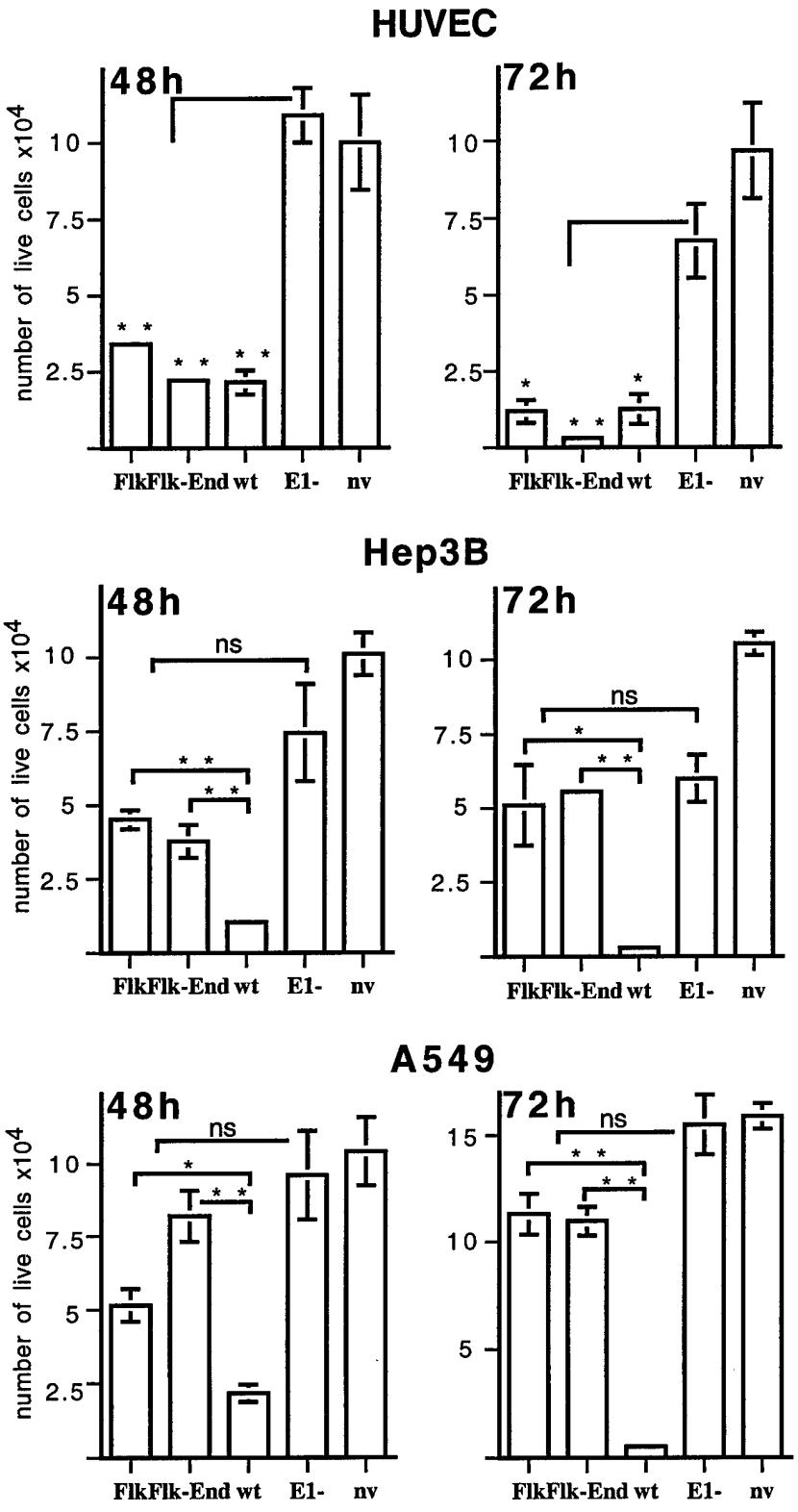

HUVEC
48h
72h
number of live cells x10^4
10
7.5
5
2.5
FlkFlk-End wt E1- nv
Hep3B
48h
72h
ns
A549
48h
72h
15

Figure 5

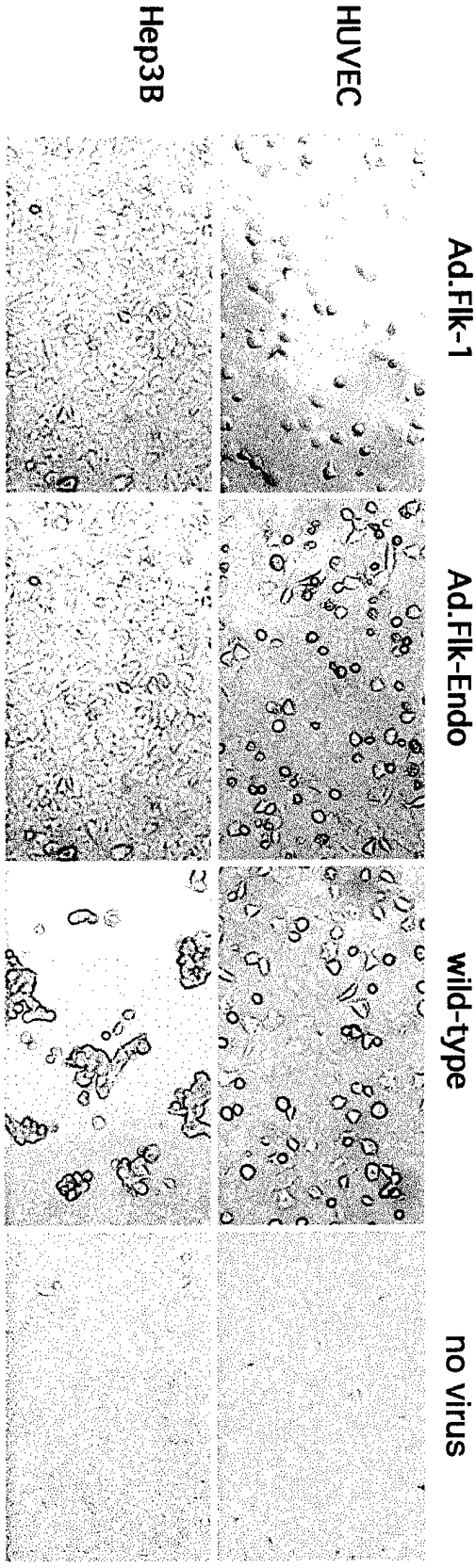
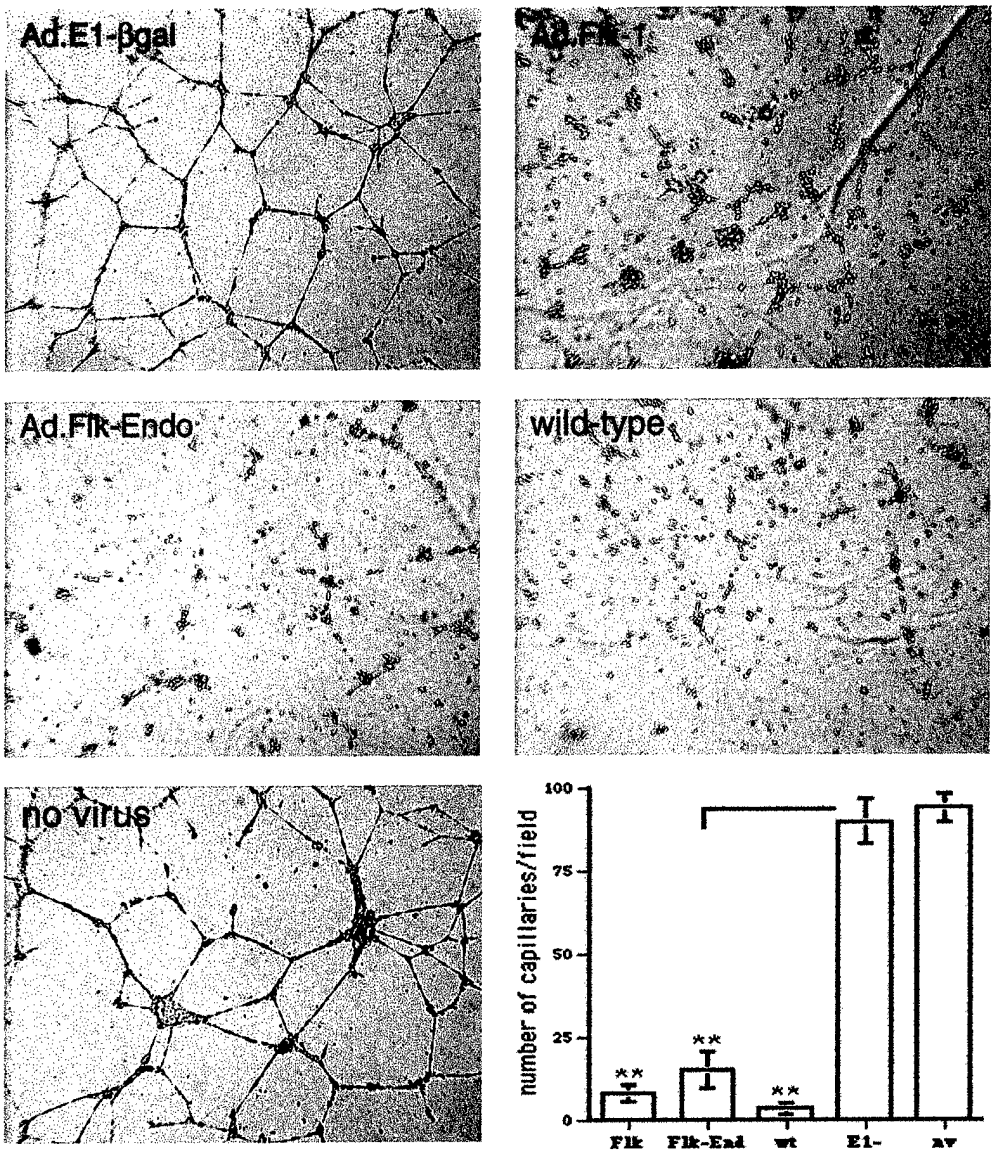


Figure 6


Ad.E1-βgal
Ad.Flk-1
Ad.Flk-Endo
wild-type
no virus
number of capillaries/field
100
75
50
25
0
**
**
**
Flk
Flk-End
wt
E1-
av

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Articles:

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* equal contribution

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2. Sauter BV, Roy Chowdhury N, Roy Chowdhury J. Classification and causes of jaundice. In: UpToDate in Gastroenterology and Hepatology, Rose, BD (Ed), UpToDate, Wellesley, MA, 2000.

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4. Sauter BV, Roy Chowdhury N, Roy Chowdhury J. Diagnostic approach to the patient with jaundice. In: UpToDate in Gastroenterology and Hepatology, Rose, BD (Ed), UpToDate, Wellesley, MA, 2000.
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N.B.: *UpToDate* is a CD ROM book aimed at the "intelllectually oriented subspecialist" and is more detailed than general medical texts. It is updated three times a year, and, therefore, it includes the cutting edge of the current medical research of a specific field.

6. N. Roy Chowdhury, B. Sauter, and J. Roy Chowdhury. Bilirubin metabolism and jaundice. Published by the American College of Gastroenterology: "Annual postgraduate and board review course: Review of Gastrointestinal Structure and Function". Chicago, October 1997.

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